Assessing efficacy and molecular mechanisms of curcumin in targeting cancer stem-like cells in colorectal cancer

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Abstract

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Curcumin inhibits the proliferation of chemotherapy-resistant cancer stem-like cells in cell lines but whether this contributes to its chemopreventive activity is unknown. This study aims to determine whether curcumin modulates the growth and expansion of colorectal stem-like cells in primary adenoma and carcinoma tissues, and in vivo using a patient-derived xenograft, then to elucidate a possible mechanism of action.

Colorectal tissue obtained post-operatively (normal n=32, adenoma n=6, carcinoma n=40) was FACS profiled for markers of stem-like cells, aldehyde dehydrogenase (ALDH) activity and CD133 expression. The percentage of cells with ALDH\textsuperscript{high} activity was 11.8±1.8, 4.6±0.7 and 2.8±0.4 in adenoma, normal and carcinoma tissues, respectively. Equivalent values for CD133 expression were 1.2±0.6, 0.5±0.2 and 7.7±1.8%. To assess in vitro activity, single cells from adenomas and carcinomas (three patients each, in triplicate) were cultured as spheroids with clinically achievable curcumin concentrations. Curcumin significantly reduced adenoma and carcinoma sphere number, compared to controls for all patient samples, with a U-shaped dose-response in >50% of cases. Sphere size was also impaired at concentrations >1µM.

Curcumin (0.2%) consumption in NOD/SCID mice injected (s.c) with cancer stem-like cells was associated with significant delay in time to palpable tumours, increased survival and reduced rate of tumour growth. There was also a ~60% reduction in proportion of ALDH\textsuperscript{high} cells in tumours from curcumin treated mice compared to controls (p<0.05).

Curcumin (0.1, 1µM) treatment of Caco2 cells caused a significant decrease (p<0.01) in Nanog expression, an embryonic stem cell transcription factor, in cancer stem-like cells specifically. A protein pull-down assay confirmed the interaction between curcumin and Nanog. Curcumin reduced (p<0.01) Nanog phosphorylation in cancer stem-like cells which may destabilise the protein, leading to reduced levels.

These results indicate that clinically achievable concentrations of curcumin target stem-like cells in colorectal adenomas and carcinomas, which may contribute to anti-cancer efficacy in humans.
Acknowledgements

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<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ACF</td>
<td>Aberrant Crypt foci</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BD</td>
<td>Beckon Dickinson</td>
</tr>
<tr>
<td>BDMC</td>
<td>Bis Methoxy curcumin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>COG</td>
<td>Curcumin O-glucuronide</td>
</tr>
<tr>
<td>COS</td>
<td>Curcumin O-sulfate</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CST</td>
<td>Cytometer set-up and tracking</td>
</tr>
<tr>
<td>CXCR-1</td>
<td>Chemokine receptor 1</td>
</tr>
<tr>
<td>DCAMKL-1</td>
<td>Double cordin CaM kinase like-1</td>
</tr>
<tr>
<td>DMSO</td>
<td>Di methyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Embryonic carcinoma</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EG</td>
<td>Embryonic germ cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>HMG</td>
<td>High motility group</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibiting factor</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non obese diabetic severe combined immune-deficient</td>
</tr>
<tr>
<td>NSAIDS</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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List of Publications


List of Conference Abstracts


1. **Chapter one: Introduction**

1.1. **Cancer Statistics**

Cancer is one of the leading causes of death throughout the world. Global cancer incidence has increased over the past decade while the mortality rate from all types of cancer has changed little over the past few years despite improvements in cancer treatment and screening [1]. In the U.K. cancer incidence has increased by 25% from 1977 to 2006, with a 14% increase in men and a 32% increase in women. It is currently estimated that 1 in 3 people in the U.K will develop some form of cancer during their lifetime. According to U.K cancer incidence statistics for 2006, breast cancer is top of the chart, accounting for 16% of all cancers (excluding non-melanoma skin cancer), lung cancer is the second most common (13%) followed by colorectal cancer (13%). Cumulatively, breast, lung, colorectal and prostate cancer account for more than half (54%) of all cancers diagnosed in the U.K (Figure 1.1). Although cancer incidence has shown a rising trend, mortality resulting from all cancers in the U.K has decreased by almost a fifth between 1978 and 2007, with the exception of certain malignancies including pancreatic and liver cancer, which have increased by 6% and 21% respectively, between 1998-2007. However, still 27% (1 in 4 people) of all deaths in the U.K in 2007 were due to cancer. The major contributor to cancer mortality in 2007 was lung cancer (22%, 1 in 5), followed by colorectal (10%, 1 in 10) and breast cancer (8%, 1 in 12) (Figure 1.2). The increased cancer incidence can be attributed to people adopting a less healthy lifestyle (smoking, high fat diets), and exposure to other environmental risk factors. Presently, it is estimated that 30% of all deaths due to malignancy in western countries are caused by smoking, 30%-40% are due to nutritional factors, calorie intake, and obesity, 10% relates to reproductive and hormonal problems, whilst another 10% can be attributed to genetic defects. The remaining risk is linked with exposure to biological (viruses) and other environmental carcinogens [2, 3].

Where observed, the decreases in mortality rate of certain cancers in western countries are mainly due to changes in lifestyle and/or advances in cancer screening; the decrease in cervical cancer mortality for example can be ascribed to well-established screening programs and improved lifestyle [3]. For stomach and endometrial cancer the decline is
possibly caused by dietary changes (e.g. lower intake of salts) and reduced use of therapeutic estrogens, respectively [3]. According to estimates of cancer incidence in the U.S the rates of lung cancer in men and colorectal cancer in both men and women are declining, whereas other malignancies including breast and prostate cancer are on an increasing trend. The possible reason for such increases could be improved screening in the form of prostate specific antigen testing for men and mammography for women, leading to a greater proportion of cancers being detected. Furthermore, the use of hormone replacement therapy may contribute to the increased occurrence of female breast cancer [4].

The overall incidence and mortality rates, as revealed by current statistics, show that cancer has now surpassed heart disease as the major cause of death for people under the age of 85 [4]. This suggests that improved diagnostic and therapeutic approaches for cancer management have been effective in some circumstances but not for cancer as a whole. As a result, cancer remains a leading cause of death, which highlights the need for new strategies, particularly preventive measures such as chemoprevention.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Percentage</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>Non-Hodgkin Lymphoma</td>
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</tr>
<tr>
<td>Malignant Melanoma</td>
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<tr>
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<td>Kidney</td>
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<td>Oesophagus</td>
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<td>Stomach</td>
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<td>Pancreas</td>
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<tr>
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<tr>
<td>Liver</td>
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<tr>
<td>Cervix</td>
<td>1%</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>1%</td>
</tr>
<tr>
<td>Other</td>
<td>2%</td>
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</table>

**Figure 1.1. The 20 most commonly diagnosed cancer in persons in the UK.** Data taken from Cancer Research U.K (http://info.cancerresearchuk.org/cancerstats/).
1.2. Cancer Management

As cancer is a class of diseases arising out of multiple factors, there is no single treatment regimen. The selection of cancer treatment is dependent on two major aspects, the site of the primary tumour and histopathological characterization of the tumour [5]. Presently, there are various options for cancer management which include surgery, radiotherapy, chemotherapy, immunotherapy, hormonal therapy and gene therapy, depending on the type of cancer. Conventional use of chemotherapy or radiotherapy presents the unresolved problem of damaging normal cells, or in other words, is not specific enough to selectively target tumour cells. This has given way to the development of alternative or more targeted treatment strategies, which have received a great impetus in the past decade. Targeted therapy includes use of anticancer agents which have a validated effect on targets critical for tumour growth [6]. However, use of targeted agents is not without toxicities. Cetuximab and Panitumunab, for example, which are used in the treatment of colorectal cancer and target the epidermal

Figure 1.2. The 20 most common causes of death from cancer in UK men and women. Data taken from Cancer Research U.K (http://info.cancerresearchuk.org.cancerstats/).
growth factor receptor (EGFR), are associated with common side effects that include allergic and dermatologic reactions [7]. Bevacizumab, a monoclonal antibody that targets vascular endothelial growth factor (VEGF) and is used for the treatment of a variety of cancers, including colorectal, has been associated with increased risk of thromboembolic complications, mostly in elderly patients above 65 years where the risk of such complication is almost doubled [8].

Treatment of cancer in patients with advanced stage metastases, or cases of unknown primary tumours with new generation cytotoxic chemotherapeutic agents has made little or no difference to patient survival or quality of life. Typically, the survival rates range from 7 to 11 months for patients with these advanced disease states as evident through various clinical studies in Europe and America [9, 10]. As these advanced malignancies present with such a dismal prognosis, it is clear that the focus should be on early diagnosis and treatment of these diseases prior to systematic dissemination or even better, to prevent neoplastic transformation altogether [11].

Recent advances in tumour biology have identified a certain subset of cells within the tumour that have the ability to reform tumours even after chemotherapy. This implies that cancer therapies may be able to kill the bulk of the tumour population, but are not as effective at killing this subset of cells, which proliferate to regenerate tumours [12]. The population that drives tumour growth and recurrence has been termed stem-like or cancer initiating cells [13, 14]. Their discovery has prompted the development of treatment or prevention approaches that are specifically targeted against cancer stem-like cells. Although cancer stem-like cell targeted therapeutic or prevention strategies are at a very early stage of development, there are currently some clinical trials that are designed to develop potential anticancer drugs targeting cancer stem-like cells in various cancers. Phase I clinical studies with Metformin, a well-established anti-diabetic drug have shown activity against cancer stem-like cells in pancreatic cancer [15]. Metformin is also currently being used in a phase I trial to assess the impact of pre-treatment with metformin on colorectal cancer stem-like cells. Similarly, for breast cancer, a pre-clinical study with primary human breast cancer xenografts have indicated that blockade of chemokine receptor 1 (CXCR-1) could sensitise the breast cancer stem-like cells and thus could enhance the efficacy of standard chemotherapy [16]. Therefore,
preventive or therapeutic approaches targeting stem-like cells provide a promising cancer treatment or prevention strategy.

1.3. **Carcinogenesis and chemoprevention**

1.3.1. **Carcinogenesis**

Carcinogenesis is a multi-step process which comprises an abnormal cellular phenomenon of differentiation, apoptosis or both, as a consequence of prolonged and complex interactions between genetic factors and environmental stress [11, 17]. Carcinogenesis has traditionally been divided into three distinct sequential phases of development (Figure 1.3) with the first step being initiation, an irreversible process involving DNA damage and mutation induction [18]. This stage encompasses the uptake of carcinogenic agents and their distribution into organs or tissues where metabolic activation and detoxification can occur [19]. The covalent interaction of these reactive species with target cell DNA can lead to the generation of DNA damage, which includes DNA adducts, oxidised lesions and strand breaks [19]. As DNA repair and damage are coupled to each other, the presence of DNA adducts does not necessarily guarantee the generation of mutations. However, if adducts are not repaired, DNA replication might facilitate alteration of DNA sequences leading to accumulation of permanent DNA mutations [20]. The second step is cancer promotion, which is reversible and is believed to include epigenetic mechanisms [18] that lead to the development of pre-malignant tumour cells [18]. This phase encompasses the loss of a wide range of cellular functions which can lead to increased cell proliferation, the alteration of apoptosis regulatory proteins and loss of cell cycle check points. The third step is cancer progression caused by mutagenic and epigenetic changes which result in the formation of neoplastic cells with increased proliferative activity and metastatic potential [18]. As the development of this multistage disease may span a long period, there are many opportunities to interfere with the process, therefore, intervention strategies aimed at reversing, delaying or preventing the progression of cancer are currently being explored with much interest (Figure 1.3).
1.3.2. Chemoprevention

As lifestyle and environmental factors are causally associated with carcinogenesis, one possible preventive strategy would be avoiding exposure to environmental risk factors by monitoring work places, minimising the intake of dietary carcinogens and improving public health [11]. However, the specific environmental risk factors associated with many epithelial cancers are poorly defined [11] [21]. Genetic mutations that predispose individuals to cancer could also be targets for interventions. However, this approach is limited as only small populations with common malignancies have well defined hereditary cancer syndromes associated with known patterns of genetic mutations [11].

An alternative promising approach towards cancer prevention is chemoprevention. Chemoprevention is a collective term for the use of dietary or pharmaceutical interventions designed to inhibit, delay or reverse the process of carcinogenesis [22]. The knowledge of molecular mechanisms underlying the development of cancer through its different stages (initiation, promotion and progression) and identifying compounds that modulate these molecular mechanisms are important for the development of potential chemopreventive agents [22]. A key consideration in the use
of chemopreventive agents is that long-term use in healthy people demands that these compounds have either no or severely limited toxicity.

Given the long-term nature of interventions and large scale of the trials needed to assess efficacy, the identification of suitable biomarkers is vital for the clinical development of chemopreventive agents to help identify the optimum dosing schedule and for monitoring their effectiveness in individuals [23]. Various models of cancer such as the Vogelstein colon cancer model [24], explain the intricate pathways involved in the manifestation of specific forms of cancer and hence provide a valuable source of information for identifying potential biomarkers of efficacy that may be modulated by interventions. Establishing early biomarkers of efficacy in the process of carcinogenesis identifies targets which novel chemopreventive agents can be tested against [25].

The vast majority of chemopreventive agents currently under investigation are multi-targeted, which makes it difficult to identify their key mechanisms required for anticancer activity in humans; consequently these are generally poorly defined. There is however, a wealth of preclinical data highlighting a wide array of pathways and targets affected by chemopreventive agents, which has traditionally enabled them to be broadly categorised as either blocking or suppressing agents [26]. Blocking agents prevent carcinogens from reaching target sites or from subsequent interaction with cellular macromolecules such as DNA, RNA or proteins [19]. They may achieve this by altering the expression and/or activity profile of phase I and II drug metabolising enzymes, up-regulating DNA repair or scavenging reactive oxygen species [27]. Even if the initial DNA damage has been induced, blocking agents may still be effective in limiting further accumulation of DNA damage. [27]. Examples of blocking agents which act via induction of phase II detoxifying enzymes include isothiocyanates and polyphenols [28].

Suppressing mechanisms of action are more prominent in the promotion and progression phase of carcinogenesis. Suppressing reduces proliferation of initiated cells, alters gene expression and restores apoptosis to normal levels, thereby preventing accumulation of damaged cell populations [27]. Experimental evidence exists in support of certain non-steroidal anti-inflammatory drugs (NSAIDs) and dietary
components being effective in targeting abnormal cells at the later stages of the carcinogenesis pathway. The net effect of these compounds is altered transcriptional regulation leading to reduced expression of genes key to the promotion and progression phases of carcinogenesis [27]. Examples of suppressing agents include the NSAID aspirin and curcumin, a constituent of turmeric, which can both act by inhibiting the metabolism of arachidonic acid and thereby prostaglandin production, through effects on COX-2 [27, 29]. Due to the multi-targeted nature of chemopreventive agents many inhibitors act both as blocking and suppressing agents with the potential for complementary effects [26, 27].

1.4. Pre-clinical development of chemopreventive agents

Preclinical assessment of potential chemopreventive agents provides a strong rationale for their clinical development. The National Cancer Institute preclinical chemoprevention agent screening program is a prime example of a methodical approach towards selection of potential agents for clinical trials through a series of in-vitro testing followed by in vivo screening [30]. The first of the sequel involves short-term pre-screening performed through various biochemical assays to identify mechanism of action of potential chemopreventive compounds. Examples of the types of assays used include carcinogen-DNA binding, prostaglandin synthesis inhibition, glutathione-S-transferase inhibition, and ornithine decarboxylase inhibition [11]. This is followed by in vitro testing of selected compounds in human-derived cell lines to predict their chemopreventive efficacy in vivo. The next stage is short term in vivo evaluation of effectiveness in models that represent the early phases of carcinogenesis. Two commonly used experimental systems are the aberrant crypt foci assay (ACF) in mouse and rat colon, and the rat mammary gland ductal carcinoma in situ assay [11, 30]. The most important part of the preclinical evaluation of a potential chemopreventive agent involves assessing its efficacy in suitable animal models that resemble, as close as possible, the in vivo situation in humans. Such models typically include use of genetically engineered rodents such as the mutant ApcMin mouse, which is used for efficacy testing pre-screened compounds that have exhibited activity against colorectal cancer [31].
The wide ranging mechanisms engaged by chemopreventive agents make a complex situation when designing clinical trials involving such agents. As recommended by the NIH, clinical chemoprevention trials are generally divided into three phases, in congruence with phased drug development strategies [32]. Phase I pilot studies constitute the pharmacodynamic and pharmacokinetic assessment of the agent. Importantly, phase I evaluation also provides scope for assessing safety and tolerance to escalating doses. Phase II trials are generally randomised, blinded and placebo controlled. These studies evaluate the dose response and toxicity profiles under prolonged exposure (preferably 3 months or longer). Potential biomarkers of efficacy relating to carcinogenesis or mechanisms of neoplastic transformation are also investigated. Phase III trials constitute the most effective stage for assessing clinical efficacy and determining the long term toxicity profile of potential chemopreventive agents, over a prolonged period of testing. These trials are usually randomised, blinded and placebo controlled. The long time frame associated with phase III trials necessitates assurances of reproducibility of the formulation administered and patient compliance [33, 34].

Clinical trials evaluating drugs for cancer treatment and agents for chemoprevention have different endpoints. Whilst for a therapeutic agent efficacy can be assessed through measurements of biological or clinical changes, such as a decrease in tumour size, currently such surrogates rarely exist in chemoprevention trials [11]. Hence, when designing clinical trials for cancer chemoprevention, certain pivotal issues must be assessed very carefully before proceeding. These include 1) determining the acceptable therapeutic index for interventions in healthy individuals, 2) thorough evaluation of existing preliminary data to rationalise trials in human subjects, 3) strategies to enhance adherence behaviour as this effects the outcome of a clinical trial; current adherence monitoring for long term trials includes self-reporting, pill counts, body fluid assays or a combination of these methods [33, 35-37], 4) assessment of cancer risk of the recruited subjects in a long-term prevention trial, and 5) identifying suitable biomarkers as surrogates for measuring preventive efficacy of the chemopreventive agent [38].
1.6. Curcumin as a chemopreventive agent

Curcumin is a polyphenolic compound which is the major biologically active constituent of turmeric, a commonly used Indian spice [39]. The efficacy of curcumin in the treatment or prevention of numerous pathophysiological conditions has been well demonstrated in a wide variety of preclinical models. These include cardiovascular disease, carcinogenesis, inflammation, and wound healing [40]. Epidemiological data also suggest that a high intake of turmeric (~1.5 g daily per person) by Asian populations could be a contributory factor for a reduced incidence of inflammatory bowel disease (almost half) and bowel cancer (one eighth) compared to western countries [40]. The cancer chemopreventive potential of curcumin has been demonstrated in numerous laboratory studies. Curcumin have shown efficacy in various animal models of cancer associated with breast, colon and pancreas [41]. In cancer, curcumin has been shown to affect numerous cellular pathways that regulate processes including cell cycle arrest, apoptosis, cell proliferation, angiogenesis and metastasis [40]. For example, curcumin has been reported to promote cell cycle arrest by dowregulating cyclin D1 expression, which is deregulated in a variety of tumours [42]. Curcumin also induces apoptosis in tumour cells by activating caspase-8, which triggers a cascade of molecular events leading to apoptosis [43] and alters the expression of genes involved in cell proliferation, metastases, invasion and resistance to chemotherapy [44]. One study demonstrated, that curcumin in colon cancer cells formed ligand with vitamin D receptor, thereby conforming to its transcriptionally active form [39]. In Caco-2 cells curcumin treatment upregulated Vitamin D receptor (VDR) target genes such as CYP3A4, CYP24, and p21 as measured by quantitative RT-PCR [39]. This implicates the vitamin D receptor as a dietary sensor of curcumin by which curcumin may mediate chemopreventive effects in colon cancer cells. The activation of VDR by curcumin in colon cancer cells required relatively high concentrations of 1-10µM. Many in vivo studies have successfully demonstrated the efficacy of curcumin as a chemopreventive agent. For example, Mahmoud and co-workers revealed that dietary administration of 0.15% curcumin decreased tumour formation by 63% in ApcMin mice; these animals represent a model of the human hereditary condition familial adenomatous polyposis and spontaneously develop intestinal adenomas as a consequence of a mutation in the Apc gene [45]. Similar effects were also reported by Perkins et al., who described a reduction in adenoma multiplicity of 39% and 40% in
ApcMin mice that received 0.2% or 0.5% dietary curcumin, respectively [31]. Curcumin also exhibits anticancer effects by reducing tumour burden in xenograft models of prostate and pancreatic cancer [46, 47]. In vivo efficacy has also been described against mammary carcinogenesis; curcumin significantly enhanced tumour free survival and reduced tumour multiplicity in BALB-neuT mice, transgenic for the neu oncogene that develops hyperplasia and invasive carcinoma at 6 and 16 weeks, respectively [48]. All these anticancer effects of curcumin make it a promising candidate for further evaluation as a chemopreventive agent.

1.7. Clinical pharmacokinetics and metabolism of curcumin

Extracted curcumin exists as a powder form that constitutes 75% of total curcuminoids. The other natural derivatives present in the mixture include 16% demethoxycurcumin (DMC), 8% bis-DMC and a small amount of cyclocurcumin [40] (Figure 1.4). Both DMC and bis-DMC are biologically active compounds [40]. Of note, the systemic bioavailability of curcuminoids is low, with plasma concentrations of less than 50 ng/mL achieved after oral ingestion of curcuminoids at doses of up to 12 g/day in humans [49]. At this high dose, curcumin levels peaked in plasma at 1-2 h following ingestion. Following lower oral doses of 4g and 8g in patient with pre-malignant lesions the average peak curcumin levels in plasma were detected at 0.51 µM and 1.77 µM, respectively [40, 50]. At an oral dose of 2 g or lower, only trace levels of systemic curcumin were detected. This low systemic bioavailability could be attributed to poor absorption, poor solubility and rapid metabolism. As such, in human and rodents models, following oral administration curcuminoids have been shown to be metabolised rapidly to curcumin O-glucuronide (COG) and curcumin O-sulfate (COS) [49]. Despite the low systemic bioavailability, curcumin can generate detectable levels in certain tissues, particularly the gastrointestinal tract when delivered orally. Administration of up to 1.2 g/kg body weight of curcumin to rats yielded a concentration of 1.8 µmol/g curcumin in colonic mucosa [51]. In patients undergoing surgery for colorectal cancer, oral consumption of curcumin at 3.6 g per day resulted in a mean level of 12.7 and 7.7 nmol/g in normal and malignant tissue, respectively [52]. Metabolites of curcumin have also been detected at nanomolar concentrations in liver tissues of patients following a dose of 3.6 g/day.
Curcumin can be considered as a relatively safe compound from a chemopreventive standpoint. Side-effects of curcumin in humans are dose related and mainly gastrointestinal that includes loose stools, bloating, reflux and discomfort [40]. Importantly adverse events may increase beyond 4 g curcumin per day in humans [40], although phase I clinical study with curcumin has demonstrated that curcumin is well tolerated in humans, upto a dose of 8000 mg/day when taken for 3 months orally [52].

Although bioavailability is a concerning issue for curcumin, it might be possible to achieve a biological effect even at low concentrations, since evidence of both local and systemic efficacy has been described in mouse models of FAP and colorectal liver metastasis [40].

![Figure 1.4. Curcuminoids and major metabolites of curcumin.](image)

Curcuminoids include bis-DMC, curcumin and DMC. The major metabolites of curcumin are the glucuronide and sulphate conjugates and these are also formed from DMC and bis-DMC.

1.8. Stem cell development

Stem cells can be defined by their exclusive properties by which they differentiate into different cell lineages and proliferate indefinitely (self-renew). The earliest embryonic stem cell that can generate a whole organism (totipotent) is the zygote. Totipotency persists from zygote to the eight cell state of morula. Subsequent blastocyst formation results in the formation of an inner cell mass (ICM) and an outer tropoblast. The ICM is no longer totipotent, but still possesses the capacity to generate various cell types of the
embryo. These cells of the ICM in the blastocyst are pluripotent embryonic stem cells, which are capable of forming the three germ layers, namely ectoderm, mesoderm and endoderm. On formation of these three germ layers, pluripotency is lost and cells become multipotent (adult stem cells that have the capacity to differentiate into cells specific to the organ in which they reside), leading to a more committed progenitor and fully differentiated cells (Figure 1.5). The properties of both adult and embryonic stem cells make them invaluable in cell replacement therapies and regenerative medicine [53]. Also, as the stem cells have potential for unlimited self-renewal, the roles of such cells in cancer are now being investigated extensively.

Figure 1.5. Development of stem cells. The earliest stem cell is a zygote (totipotent). The next stage is an 8-cell stage morula having totipotent cells. The early blastocyst consists of an inner cell mass (ICM) having pluripotent stem cells. The outer layer of cells is called tropoblast having no stem cell population. The later blastocyst stage is comprised of epiblast having a pluripotent stem cell population which gives rise to three germ layers of ectoderm, endoderm and mesoderm. The primitive endoderm and trophoectoderm cells of the blastocyst lack any pluripotency. The germ layers lead to multipotent cells which develop into progenitor and fully differentiated cells.
1.9. **Cancer Stem cells**

The classical hypothesis of carcinogenesis describes cancer development as a consequence of a multi-step phenomenon which ultimately converts a normal cell into a transformed cell that has aberrant cellular functions. There has been much debate regarding what the target cell population for neoplastic transformation is. Several studies have confirmed that accumulation of genetic mutations in normal cells over a prolonged period that act in concert, give rise to a malignant phenotype. It has been shown that in models of ascites fluid in rats, embryonic carcinoma and mouse myeloma tumours, a single tumour cell was capable of forming a new tumour with a heterogeneous progeny [54-57], thus providing strong evidence for a clonal origin of tumours. There has been an impressive body of findings suggesting that clonal expansion leads to cells harbouring genetic mutations that result in malignant transformation [58, 59]. As many tumours retain features associated with the normal surrounding differentiated epithelium, the target of such accrued neoplastic genetic mutations could be these differentiated cells [60]. However, for most terminally differentiated cells, as in case of epithelial tissues, their proliferating capacity is redundant and they are continually replaced by newly formed differentiated cells. As genetic mutations in tumours are accumulated over a prolonged time span, this suggests that differentiated cells are not the sole targets for these kinds of mutations. Furthermore, tumours have a heterogeneous population of cells exhibiting varying degrees of differentiation and transformation. Therefore, it seems unlikely that a well-differentiated target cell would give rise to progeny with multiple degrees of differentiation, even considering a small degree of dedifferentiation induced by the process of transformation [60]. Moreover, current experimental findings for many cancer cell lines suggest that a relatively large number of cells are required for tumour formation in immuno-compromised mice. This low tumour forming efficiency suggests that not all cells have the capacity to recapitulate tumour formation and there might a sub-population of tumour cells capable of driving tumour formation.

Recent advances in tumour biology, in conjunction with stem cell biology, have led to the identification of certain subsets of cells within the tumour population that are characterised as possessing properties of stem cells. Epithelial tissues are subjected to continuous remodelling and renewal in a tightly regulated fashion [61] through a
process that involves an array of specialised functional cells, including stem cells, slow proliferating cells and terminally differentiated cells [60]. As these stem-like cells are generally quiescent and possess an unlimited self-renewal capacity, along with the ability to form differentiated progeny, these cells serve as the ideal target population for neoplastic mutation accumulation. The increasing evidence of stem-like cells being involved in the process of tumour development has led to the Cancer stem cell hypothesis, which states that cancer originates from tissue stem cells or their subsequent progeny, through de-regulation of a tightly controlled self-renewal phenomenon. However, the classification of stem-like cells within cancers, as with normal adult stem cells, remains purely functional. There remains a possibility that cancer stem-like cells only imitate certain stem cell phenotypes such as self-renewal and differentiation, or represent altered early transit amplifying cells, as the precise relationship between normal stem cells and cancer stem-like cells remains poorly understood [60, 62]. Another possible origin of cancer stem-like cell is through the process of de-differentiation, which allows progenitor cells to de-differentiate and acquire the properties of a stem cell. This route has been implicated in the haematopoietic system, where some progenitor cells can acquire the ability to self-renew, which is a central feature of stem cells [63]. It is important to note that the cells of origin and cancer stem cell are distinct concepts. The cell of origin refers to the normal cell acquiring the first cancer promoting mutation whereas cancer stem cells are unique population within the tumour mass that sustains malignant growth [64].

1.10. Properties of stem cells

Stem cells are defined by specific functional attributes. The process of self-renewal is the inherent capacity of stem cells to produce new stem cells, while maintaining similar functional capacities, including an intact potential for proliferation, expansion, and differentiation, thus maintaining a steady pool of stem cells. Stem cells also exhibit the ability to give rise to a heterogeneous population of a progressively differentiated progeny, constantly replenishing short-lived tissues. Finally, stem cells possess the ability to strike a homeostatic balance by modulating the process of self-renewal and differentiation in response to environmental stimuli and genetic alterations [65]. Cancer stem-like cells resemble normal stem cells functionally in the following ways, all of which present strong support in favour of the cancer stem cell hypothesis [66].
a) self-renewal capacity

b) differentiation

c) active telomerase expression

d) activation of anti-apoptotic pathways

e) increased membrane transporter activity

In normal steady state conditions, stem cell self-renewal is governed by asymmetric cell division, which produces a copy of the stem cell itself and a differentiated lineage. During tumourigenesis, the normal asymmetric cell division may become deregulated to a symmetric cell division, which produces two identical stem-like cells, thus allowing for an expansion of the stem-like cell population (Figure 1.6). This process of stem cell expansion through an aberrant stem cell self-renewal mode may be a key early event in the development of carcinogenesis [66]. Several cell signalling pathways such as Wnt, Notch and Hedgehog have been suggested to regulate the process of self-renewal in hematopoietic, neuronal, and mammary stem cells [67, 68], and dysregulation of these pathways is associated with the development of carcinogenesis. The first evidence of aberrant Hedgehog signalling was reported in human basal skin carcinoma [69]. Recent findings also suggest a role for a deregulated Hedgehog pathway in human pancreatic, gastric, prostate and breast cancer [70, 71]. Dysregulation of Wnt signalling has been implicated in early colon cancer carcinogenesis [66], whereas alterations in Notch signalling have been observed in cervical cancer, acute leukaemia and breast cancer [72-75].
Figure 1.6. Comparison between normal and cancer stem-like cell proliferation. A normal stem cell divides through a tightly regulated process of self-renewal and differentiation, maintaining a homeostatic balance between the two routes. Accumulation of mutations can cause a normal stem cell to acquire the properties of a cancer stem-like cell in which the process of self-renewal becomes aberrant and the resulting tumour represents a heterogeneous progeny comprising cancer stem-like cells, proliferating cells and senescent or dying cancer cells. The tumour heterogeneity increases with more advanced tumour state.

1.11. Markers of cancer stem-like cells

The cancer stem cell hypothesis has opened new frontiers in stem cell biology aimed at a better understanding of the role of cancer stem cells in the process of tumourigenesis. The focal point of such scientific studies has been to purify and characterise these cancer stem-like cells to assess the validity of the hypothesis. The assessment of stem cell properties has been facilitated by the development of animal models that are efficient in defining self-renewal and differentiation properties. One such model is the NOD/SCID mouse (non-obese diabetic/severe combined immunodeficiency), which being immune-compromised, allows the in vivo functions of transplanted human cells to be assessed. To this effect, a number of groups have reported stem cell purification techniques mainly based on the differential expression of cell surface markers and have employed NOD/SCID mice as an in vivo tool to ascertain purity of isolated fractions of
cancer stem-like cells. Isolation and purification of stem-like cells from primary patient tumour tissues is followed by a battery of tests, both in vivo and in vitro, to ascertain stemness of these cells, as outlined in Figure 1.7.

The first isolation of cancer stem-like cells, based on the expression of cell surface antigens, was reported by John Dick and Dominique Bonnet, who demonstrated that human leukaemia, could be recapitulated in NOD/SCID mice by transplantation of a small population of leukemic cells with a CD34+/CD38− phenotype [76]. These cells, which were present at a frequency of < 1 in 10,000 leukaemia cells, were able to form initiate acute myeloid leukaemia in NOD/SCID mice, whereas an injection of a thousand-fold higher number of cells lacking the CD34+/CD38− cell surface marker, failed to form tumours. Moreover, tumours generated from these CD34+/CD38− cells presented histopathological resemblance to the original tumour.

Subsequent to the work on haematological malignancies, solid tumours in brain and breast were also shown to possess a subpopulation of cancer stem-like cells. In breast tumours, a small population of cells expressing CD44 and little or no CD24 showed a high tumourigenic potential [13]. As few as 100 cells of the CD44+/CD24low− phenotype were required to form tumour xenografts in NOD/SCID mice over two generations, whereas injection of thousands of cells with CD44+/CD24high phenotype failed to do so. Similarly, in brain tumours a subpopulation of cells with CD133 expression exhibited properties of cancer stem-like cells. CD133 is a surface antigen in normal neural stem cells, which is also present in stem and progenitor cells of various other tissues [77]. CD133+ cells from brain tumours displayed a capacity for self-renewal in both in vitro and in vivo systems [14, 78] and injection of as few as 100 CD133+ cells in NOD/SCID mice generated xenografts representing the exact original tumour characteristics, and could be serially transplanted. In contrast, an injection of 10^5 CD133− cells although successfully engrafted, failed to form tumours [79]. Recently, several groups have confirmed the presence of cancer stem-like cells in several other epithelial cancers including pancreatic, prostate, and colorectal (Table 1.1).
Figure 1.7. A schematic representation for testing the stem-like properties of cancer cells. Following the isolation of stem-like cells from primary tumour tissues, based on the presence of certain stem cell markers, the cells undergo a sequence of both *in vivo* and *in vitro* tests to verify their stemness. Tumours generated from xeno-transplantation in NOD/SCID mice exhibit similar morphological and molecular features to the primary patient tumour. The primary tumour characteristics are maintained in subsequent tumours generated at different passages of a serial transplantation experiment. This confirms the presence of certain cell subsets capable of self-renewing and driving the process of tumourigenesis. The differentiation capacity of cancer stem-like cells is tested by allowing spheroids derived from isolated cells to differentiate in serum containing media, without supplements of stem cell growth factors.
Table 1.1 Tumourigenicity of various cancers based on the distinctive expression of cell surface markers.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cancer stem cell phenotype based on cell surface markers</th>
<th>Tumourigenicity of stem-like cells: number of cells capable of forming xenografts in NOD/SCID mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>CD44+/CD24&lt;sup&gt;-low&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>Prostate</td>
<td>CD44+/α2β1&lt;sup&gt;high&lt;/sup&gt;/CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>500</td>
</tr>
<tr>
<td>Colon</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>CD44+/ESA&lt;sup&gt;+&lt;/sup&gt;/CD24&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>Brain</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: All experiments were done with patient derived tumour cells. Data taken from references [13][14][75][76][80][80].

1.12. Stem cells in colorectal carcinogenesis

Recently, two groups have identified the existence of colon cancer initiating cells (CC-IC) [78, 81], which are characterised by the presence of CD133 cell surface antigens. These CD133<sup>+</sup> cells express epithelial antigens BerEp4 (also known as EpCAM and ESA) but lack differentiation markers such as CK-20, which are present mostly in mature gastrointestinal cells [82]. Normal colonic tissues also contain cells with the CD133<sup>+</sup> phenotype, but at a much lower frequency compared with their cancer counterpart [82]. According to the authors, this provides evidence that normal colon stem cells may have undergone oncogenic transformations resulting in enhanced CD133<sup>+</sup> expression in colon cancer initiating cells [82]. Subcutaneous injection of just 100 CD133<sup>+</sup> cells into NOD/SCID mice generated a phenocopy of the original colon tumour, demonstrating the high tumourigenicity of these cells, which was further confirmed by successful serial transplantation in NOD/SCID mice.

Darlerba and co-workers have reported an alternative purification method for colon cancer initiating cells based on the cell surface expression profile of EPCAM<sup>high</sup>/CD44<sup>+</sup>/CD166<sup>+</sup> [83]. The selection of EpCAM and CD44 is based on their previously observed phenotype for breast cancer stem-like cells, whilst high CD166 expression, a mesenchymal stem cell marker, has been associated with poor clinical outcome in
colorectal cancer [82, 84]. Experimental evidence presented by Dalebra et al. [83] suggests that tumourigenic potential is restricted to the CD44+/CD166+ population, which represents a subpopulation of EpCAM+ cells. Based on xenograft studies the authors indicated that EpCAM together with CD44 is a more robust marker of stemness in colon cancer initiating cells than CD133+ alone [82].

The specificity of CD133 or CD44/ESA as markers of stem-like cells in colon cancer has been questioned by recent studies reporting that CD133 expression is not specific to this population, since both CD133+ and CD133− cells isolated from primary metastatic colon cancer tissue generated xenografts in mice [85]. Immunohistochemical analysis of normal mouse intestine has also revealed that CD44+ expression is not restricted to the base of the crypt (stem cell niche) only, but was also detectable in the proliferating compartment, which harbours rapidly dividing cells [86-88]. As such, the purity of stem cells in colon isolated based on either CD133 or CD44 expression remains questionable. This calls for investigation of new markers to isolate a pure stem cell population in colon tissues. Assuming that cancer stem-like cells arise from normal stem cells, the markers used to isolate normal stem cells could be used for isolation of cancer stem-like cells.

One such promising marker is aldehyde dehydrogenase (ALDH), which is a detoxifying enzyme that oxidises intracellular aldehydes [89]. The detoxifying capacity of ALDH, by protecting stem cells from oxidative insults could be a potential mechanism for long survival of stem cells [89]. ALDH is also involved in the conversion of retinol to retinoic acid which is a modulator of cell proliferation, thereby affecting stem cell proliferation. Several groups have reported the use of ALDH activity as a means to isolate stem cells. This is demonstrated in the hematopoietic system where as few as 10 cells with high ALDH activity were capable of reconstituting the bone marrow in sub-lethally irradiated mice [90]. ALDH activity has also been reported to be a stem cell marker in breast cancer [91]. Ginestier et al. showed that in vivo breast tumour formation from patient derived xenograft models in NOD/SCID mice was only possible with ALDHhigh cells, whereas the ALDHlow fraction failed to exhibit any tumour formation in NOD/SCID mice. Similarly for colorectal cancer, as few as 25 ALDHhigh cells derived from primary colon tissue were capable of forming tumours in NOD/SCID
mice, but ALDH\textsuperscript{low} cells failed to produce any tumours [89]. Huang et al. [89] and Joseph et al. [92] have also demonstrated that high ALDH activity represents a specific marker to isolate and count the number of stem-like cells and that these increase in number during the development of colorectal cancer from normal to a malignant phenotype. Thus, current experimental evidence suggests that ALDH activity is potentially a better marker of stem-like cells compared to other markers reported for human colorectal cancer.

Another promising putative intestinal stem cell marker is DCAMKL-1 (double cortin CaM kinase like-1). DCAMKL-1 is a microtubule associated kinase expressed in post mitotic neurons [93]. Experimental evidence suggests that DCAMKL-1 is expressed in single cells near the +4 position of intestinal crypts in wild-type mice at a frequency of 1 cell in 6 intestinal crypt cross sections [94]. DCAMKL-1 staining was also observed in the intestine of \textit{Apc}\textsuperscript{Min} mice, which are a model of adenoma development as these mice carry a germ line mutation in their \textit{Apc} genes and thus form spontaneous adenomas in their intestines [94]. However the staining pattern in the \textit{Apc}\textsuperscript{Min} mice differed from that of the wild-type. Although there was occasional single cell staining in the crypts, the \textit{Apc}\textsuperscript{Min} mice had an increasing trend of DCAMKL-1 staining in the villi compared to the wild-type animals. Also, the DCAMKL-1 staining in adenomas of the \textit{Apc}\textsuperscript{Min} mice was observed in the non-proliferating cells, suggesting that DCAMKL-1 positive cells represent a quiescent cell population. There was nuclear localization of β-catenin in the DCAMKL-1 positive cells within adenomas of the \textit{Apc}\textsuperscript{Min} mice, whilst the normal looking crypts had membrane β-catenin staining in the DCAMKL-1\textsuperscript{+} intestinal cells. This suggests that normal intestinal epithelium stem cells and adenoma stem cells could be distinguished based on β-catenin and DCAMKL-1 staining [94]. It was also proposed that DCAMKL-1 can be used as a viable intestinal and possibly colonic stem cell marker to test the effects of DNA-damaging agents, chemotherapeutic drugs and radiation injury using stem cell survival as the end point. This is substantiated by an extensive study by May et al. [94] where the effect of irradiation (IR) on DCAMKL-1\textsuperscript{+} cells was assessed. Briefly, the DCAMKL-1\textsuperscript{+} cells exhibited radiation-induced DNA damage along with other cells in the crypts but did not exhibit any apoptosis at a time point of 6 h following exposure to 6 Gy radiation. However, following 24 h IR exposure, the DCAMKL-1 population showed signs of apoptosis. This is in line with
previous reported studies, which suggest that there are two waves of apoptosis that occur after IR exposure. The first wave is p53-dependent and occurs at 4-6 h and the second wave (p53-independent) occurs at 24 h post IR. The second is thought to primarily affect the stem cells [95, 96], which is consistent with the observation by May et al.

1.13. Curcumin and stem cell pathways
Normal stem cells and cancer stem-like cells share the ability to self-renew; therefore, it is not surprising that many pathways involved in stem cell renewal are also associated with carcinogenesis. Wnt, Hedgehog, and Notch pathways play a pivotal role in normal stem cell development and regulate stem cell self-renewal [63]. Also, these pathways are important determinants of cancer stem-like cell self-renewal. Therefore targeting the stem-like cell through these cellular pathways with potential anticancer agents could be an exciting avenue to develop both cancer prevention and treatment strategies. Figure 1.8 summarizes the effects of curcumin on various cellular pathways.

1.13.1. Effect of curcumin on Wnt, Hedgehog and Notch
Recent evidence supports the idea that the Wnt signalling pathway is involved in the self-renewal of stem cells in normal and cancer tissues derived from the colon, breast, prostate and skin [97]. Activation of canonical Wnt signalling together with other pathways, for example those involving Akt, a serine/threonine-specific protein kinase which plays a key role in multiple cellular processes, have been suggested to regulate stem cell self-renewal in breast cancer [98]. Curcumin (at 5 µM) has been shown to inhibit Wnt signalling in MCF-7 breast cancer cells by 50% and has also been implicated in attenuating Wnt/β-catenin signalling in colon cancer cell lines [99, 100]. A recent report has revealed that curcumin in combination with piperine exerts chemopreventive effects in breast cancer by targeting stem cell populations through inhibition of Wnt signalling [101]. In HCT 116 colon cancer cells, curcumin impaired Wnt signalling and cell-cell adhesion pathways resulting in G2/M arrest and apoptosis [99]. In addition, the naturally occurring curcumin analogues demethoxycurcumin [DMC] and bisdemethoxycurcumin [bis-DMC] inhibited Wnt signalling by decreasing expression of the transcriptional coactivator p300 [100]. Some studies propose that curcumin can target cancer stem-like cells through inhibitory effects on the Hedgehog
signalling system. This pathway is a key regulator of organ development, and has been implicated to play a role in regulating the self-renewal characteristics of pancreatic, glioma and leukaemic cancer stem-like cells [102, 103]. In support of this notion, cyclopamine, a steroid based hedgehog inhibitor, has been reported to prevent the self-renewal of glioma initiating cells in a sphere formation assay [104]. Similarly, loss of the hedgehog receptor ‘smoothened’ resulted in dysfunctional self-renewal of haematopoietic stem cells and decreased chronic myelogenous leukaemia induction by the BCR-ABL1 oncoprotein [103]. Curcumin has been shown to target hedgehog signalling in prostate cancer. The hedgehog pathway is activated by ‘smoothened’, a G-coupled receptor protein that activates the Gli transcription factors (Gli1, Gli2 and Gli3) which control the transcription of hedgehog target genes. In this context, curcumin caused a decrease in expression of Gli mRNA in TRAMP-C2 prostate cancer cells [105], thus inhibiting the hedgehog pathway. Similar effects were seen in prostate tumours of TRAMP mice administered curcumin [105]. Nano-particular curcumin at a dose of 25 mg/kg, in combination with the chemotherapeutic drug gemcitabine completely abrogated metastasis in a preclinical mouse model of pancreatic cancer [106], whereas gemcitabine alone failed to exert such effects. Furthermore, in brain tumour cell lines there have been suggestions that blocking the hedgehog pathway could be a potential mechanism of action through which nano-curcumin targets brain cancer stem-like cells [107].

The Notch signalling pathway controls cell proliferation and apoptosis related to organ development [108]. Recent studies indicate that Notch could be indirectly involved in the aberrant self-renewal of cancer stem-like cells of breast, oesophageal and pancreatic origin [108, 109]. Curcumin at 5 and 10 µM down-regulated Notch-1 leading to apoptosis via inactivation of the transcription factor NFκB in pancreatic cancer cell lines PANC-1 and BxPC-3, at the mRNA and protein levels [109]. In oesophageal cancer cells, curcumin inhibited Notch-1 activation by down-regulation of critical components of gamma secretase complex proteins [110]. Curcumin-mediated inhibition of Notch-1 was confirmed by reduced expression of Notch-1 specific microRNAs such as miR-21 and miR-34a, and enhanced expression of tumour suppressor microRNA let7a [110].
1.13.2. Effects of curcumin on Signal Transducer and Activator of Transcription (STAT) signalling relevant to cancer stem-like cells

STATs are a group of transcription factors that play an important role in communicating extracellular signals generated by cytokines and growth factors from the cytoplasm to the nucleus [111-114]. Activation of STATs by phosphorylation leads to their dimerization and subsequent translocation into the nucleus. STATs regulate the expression of genes critical for cell cycle progression, proliferation, invasion and survival [114]. Constitutive activation of STAT3 and STAT5, two of seven STATs identified so far, has been linked with multiple myeloma, lymphomas, leukaemia and some solid cancers [43] including colon cancer [115]. Recent findings suggest that STAT3 activation is essential in the maintenance of stem-like cells in certain solid tumours. In colon cancer, stem-like cells characterised biochemically by high activity of the enzyme aldehyde dehydrogenase (ALDH) and the expression of the transmembrane glycoprotein CD133 (see below), demonstrated STAT3 phosphorylation, implying that STAT3 is activated in the stem-like cell population [116]. In primary breast tissues, cancer stem-like subpopulations of cells, as reflected by the presence and absence of the glycoproteins CD44 and CD24, respectively, had significantly higher levels of phosphorylated STAT3 than cells bearing a non-stem-like phenotype [117]. Curcumin has been demonstrated to inhibit IL-6-induced STAT-3 phosphorylation and its subsequent nuclear translocation in cultured multiple myeloma cells [118]. Furthermore, curcumin targeted both the constitutive and the IL-6-inducible STAT3 pathway in head and neck squamous cell carcinomas in vitro [118]. It also inhibited STAT5 mRNA expression and down-regulated its activation in primary patient-derived myelogenous leukaemia and K562 leukaemia cells [119]. Curcumin and the synthetic curcumin analogues GO-Y030 and FLLL32 inhibited STAT3 phosphorylation and the expression of downstream target genes such as cyclinD1, survivin and BcL-XL in the stem-like population of colon cancer cell lines [43, 120].

1.13.3. Effects of curcumin on interleukin-8 relevant to cancer stem-like cells

Interleukin-8 is a chemokine with a wide range of functions, including activation of human neutrophils, chemotaxis, expression of cell surface adhesion molecules [121] and the regulation of angiogenesis [122]. Recent evidence suggests that IL-8 is involved in the self-renewal of cancer stem-like cells [123]. IL-8 is over-expressed in breast
cancer and linked with a poor prognosis [123]. IL-8 signalling depends on interactions with the cell surface G protein-coupled receptors (GPCRs) CXCR1 and CXCR2. Blocking these receptors using a non-competitive allosteric inhibitor repartaxin, increased the ability of the cytotoxic anticancer drug docetaxel to reduce tumour size in breast cancer xenograft-bearing mice [16]. In the same study, an in vivo functional assay that consisted of re-implanting cells derived from repartaxin treated tumours into secondary NOD/SCID mice, was used to assess the self-renewal capacity of cancer stem-like cells that remained after treatment. Repartaxin successfully reduced tumour growth in the secondary recipients [16]. Another recent study showed that IL-8 plays an important role in maintenance of the cancer stem-like population within primary breast cancer tissue; inhibition of CXCR1/2 by a small molecule antagonist blocked IL-8 activity in cancer stem-like cells and enhanced efficacy of the dual tyrosine kinase inhibitor lapatinib in Her-2 positive cancers, assessed through a patient-derived mammosphere forming assay [123]. Curcumin has been reported to inhibit IL-8 production by tumour cells and supress signal transduction via the GPCRs [43]. In pancreatic cancer cells curcumin attenuated the constitutive production of IL-8 and reduced NFκB activity [121]. In HCT116 colorectal cancer cells, curcumin reduced neurotensin-induced expression of IL-8 in a dose-dependent manner, a gastrointestinal hormone that stimulates cell proliferation in colon and pancreatic cancers which possess high-affinity neurotensin receptors [124]. Although all of these findings provide indirect evidence for the notion that curcumin can affect IL-8 and its receptor signalling in cancer cells, a more comprehensive experimental approach is warranted before firm conclusions as to the ability of curcumin to affect cancer stem-like cells via IL-8 inhibition can be drawn.
Figure 1.8. Effects of curcumin on different pathways germane to the self-renewal of cancer stem-like cells. Evidence suggests that curcumin can inhibit signalling through pathways involving Notch, Wnt, Hedgehog, STAT and interleukins, which are all implicated in the process of carcinogenesis. These pathways are also responsible for the self-renewal of stem-like cells in certain malignancies.

1.14. Mediators of pluripotency

Several studies have indicated that various extrinsic factors are necessary for maintaining the pluripotency of stem cells. These factors have been elucidated for both murine and human embryonic stem cells (ES). In mouse ES in vitro, activation of signal transducer STAT3 by leukaemia inhibiting factor (LIF) has been shown to support the undifferentiated state of ES cells [125]. Similarly, BMP-4 (bone morphogenetic protein) in the presence of LIF enhances the self-renewal of mouse ES cells by activating Id (inhibition of differentiation) genes [53, 126]. However, in certain culture conditions that facilitate self-renewal of mouse ES cells, it has been proposed that LIF is not sufficient for maintaining the pluripotency of the ES cells [127, 128]. These findings suggest that maintenance of pluripotency is a complicated phenomenon that involves a highly co-ordinated participation of several factors.
The extrinsic pathways (LIF/STAT3) do account for the undifferentiated states of ES cells. These pathways eventually lead to regulation of genes resulting in pluripotency [53]. One such gene is Oct4, a POU domain containing transcription factor [129]. The role of Oct4 in maintaining pluripotency is substantiated by the fact that its absence, both *in vitro* and *in vivo*, causes the pluripotent cells (epiblast) to revert to the trophoblast lineage [129], implicating a crucial role for Oct4 during early embryonic development. Surprisingly, Oct4 overexpression causes differentiation of ES cells [130], suggesting that a steady state level needs to be maintained to support pluripotency. However, Oct4 is not the only transcription factor involved in the self-renewal of ES cells, as evidenced by the fact that upon LIF withdrawal, Oct4 on its own was not sufficient to prevent differentiation of ES cells [53]. This indicates that other factors, namely Nanog and Sox2, are involved in maintaining pluripotency.

Nanog is a homeodomain-containing transcription factor [53] that has been implicated in preventing the differentiation of epiblasts (pluripotent stem cells) into primitive endoderm, thus maintain the pluripotency of ES cells in the inner cell mass (ICM) of the blastocyst [131]. Additionally, recent studies have shown that Nanog has two potent transcriptional activation domains [132, 133], suggesting that Nanog can function as a transcriptional activator. Nanog mRNA is first detected during embryogenesis in the morulae, and is then confined to the ICM and disappears from the trophectoderm in early blastocyst stage [53]. In the later blastocyst stage Nanog expression is strictly confined to the epiblast, accounting for the pluripotency of these cells. In pluripotent cell lines such as EG (embryonic germ cells) and EC (embryonic carcinoma), Nanog mRNA is enriched [134], whilst differentiation of these cells causes down regulation of Nanog [53]. A high level of Nanog can maintain pluripotency in mouse ES cells independent of LIF, suggesting that Nanog might be a major downstream effector of extrinsic factors [131, 135]. Therefore, the functional evidence in ES cells suggests that Nanog plays a central role in maintaining the pluripotency of these cells.

A third transcription factor with a functional role in maintaining pluripotency is Sox2, which belongs to the Sox family of proteins, having a highly conserved DNA binding domain HMG (high motility group) [133, 134, 136]. Although not yet characterised to
the same extent as Oct4 and Nanog, Sox2 appears to play a crucial role in the regulation of cell fate and its interaction with Oct4 provides a vital step in inducing pluripotency (iPs) which involves reprogramming of somatic cells to become induced pluripotent cells, closely resembling embryonic stem cells [137].

1.14.1. Transcriptional network involving Oct4, Sox2 and Nanog in the regulation of pluripotency

Individually, the functions of Oct4, Sox2 and Nanog in maintaining pluripotency have been studied. However, these transcriptional factors do not act in isolation to regulate the process of self-renewal and thus maintain pluripotency of ES cells; instead an interactive transcriptional regulatory network between Oct4, Sox2 and Nanog provides an essential requirement in the maintenance of pluripotency. Evidence of such interaction has been demonstrated through the induction of pluripotency by retroviral introduction of four critical genes in somatic cells, sometimes referred to as Yamanaka factors, Oct4, Sox2, Klf4 and c-Myc or another cocktail of Oct4, Sox2, Nanog and LIN28 [134] [138]. Subsequently, it has been shown that altering the culture conditions by adding valproic acid (a histone deactylase inhibitor) enhanced the efficiency of inducing pluripotency and also could introduce pluripotency using just Oct4 and Sox2 [139]. This further exemplifies the vital role that Oct4 and Sox2 plays in a complex transcriptional regulatory network.

The direct protein-protein interaction of Oct4 and Sox2 has been implicated in the regulation of a pluripotency transcriptional network. It has been shown that Oct4 and Sox2 proteins act to regulate their own transcription and also the expression of other key genes such as Nanog [140] (Figure 1.9), and Zfp42/Rex1 [141-143], which is a direct target of Nanog. As such, the whole pluripotency transcription factor complex could involve Nanog homodimers [144] and Oct4-Sox2 heterodimers [140] (Figure 1.9).

Although the exact regulatory mechanisms of Oct4 have not yet been elucidated, Sox2 may play a crucial role [136]. Oct4 has been shown to suppress its own expression when overexpressed [145] (Figure 1.9), which creates a negative feedback loop that counters the effects of Sox2 and Nanog, thus fine tuning the expression levels of Oct4 in
undifferentiated stem cells [145]. This fluctuating level of Oct4 creates a biphasic regulation of Nanog [134]. A low level of Oct4 up-regulates Nanog expression whereas a high Oct4 level down-regulates Nanog expression [129] (Figure 1.9).

Through Chromatin immunoprecipitation and DNA microarray analysis, Boyers et al. elucidated the DNA binding domains of Oct4, Sox2 and Nanog [146]. The striking finding of this work was the co-occupancy of these three transcription factors in the same gene region. There was a 50% congruency between Oct4 and Sox2 binding genes. Furthermore, Nanog bound to over 90% of the target genes promoter bound individually by Oct4 and Sox2. A total of 352 genes were shown to be simultaneously bound by Nanog, Oct4 and Sox2 in the undifferentiated human ES cells. The study conducted by Boyers et al. [146] also confirmed that these three transcription factors bound to their own promoter regions, creating an interconnected auto-regulation loop to maintain the pluripotency of ES cells [53].

1.14.2. Potential role of the Nanog pathway in cancer

The cancer stem cell hypothesis indicates that cancer stem-like or initiating cells possess certain characteristics that are similar to normal stem cells, especially the self-renewal phenomenon, which could be the driving factor for tumour initiation and growth. Mounting evidence suggests that ES cell self-renewal and pluripotency genes, including the three main transcription factors Oct4, Sox2 and Nanog, could play a vital role in driving the process of oncogenesis [147]. Functional evidence supporting the involvement of Nanog in promoting stem cell characteristics has been well documented in prostate cancer. For example, isolated cancer stem-like cells identified by a CD44\textsuperscript{high} phenotype in the DU145 prostate cancer cell line exhibited a 10-fold higher expression of Nanog compared to the bulk cell population [147]. Furthermore, Nanog knockdown by siRNA inhibited the clonogenicity of both LNCaP and MCF7 prostate and breast cancer cells respectively [148]. A more comprehensive knockdown of Nanog by ShRNA in prostate cancer (Du145, LPC4, LPC9 and HPCa18), colon cancer (Colo320) and breast cancer (MCF7) cell lines consistently impaired tumour formation in mouse xenografts [148]. In addition, in vitro clonal growth of primary patient-derived prostate cancer cells was inhibited strongly by Nanog-shRNA, whereas Oct4-shRNA had a lesser effect [148]. Nanog-shRNA mediated knockdown also decreased the size
and number of spheres in the primary prostate cancer cells and impaired the sphere forming capacity of xenograft-derived cells [148]. Overexpression of Nanog correlated with drug resistance in MCF-7 cells and tumour regeneration in castrated models of NOD/SCID-γ mice injected with Nanog overexpressing LNCaP cells [147]. Nanog overexpression also upregulated ALDH1 activity in breast and prostate cancer cells and increased the expression of stem cell genes, including Oct4 and Sox2 [147]. In the gastric cancer cell line MKN-45, the stem-like population characterised by CD44\textsuperscript{high} staining exhibited an overexpression of Oct4, Sox2 and Nanog, coupled with a higher sphere forming capacity [149]. In oral carcinoma there was a significant overexpression of Nanog and Oct4 in cisplatin resistant oral squamous cell carcinoma (OSCC) patients, assessed through tissue immunohistochemical staining in a panel of chemo-resistant OSCC patient samples. In OC2, an established oral carcinoma cell line, sphere-forming cisplatin resistant cells were shown to express higher levels of Nanog and Oct4 [150]. Using HCT116 colorectal cancer cells, a stable clone of Nanog expressing cells (Nanog+ve) were able to generate tumours in NOD/SCID mice, whereas injection of a control population (Nanog-ve) led to either delayed or no tumour growth [151]. In the same study, \textit{in vitro} sphere forming capacity was confined to the Nanog +ve population in both the HCT116 cells and another colon cell line, SW620. Immunohistochemical tissue staining of tumour samples from patients with nasopharyngeal carcinomas has revealed an over expression of Oct4, Sox2 and Nanog protein compared to normal tissues. Oct4 and Nanog, but not Sox2 expression, had a strong correlation with poor clinical outcome in these patients [152].

Overall, the evidence for Oct4, Nanog and Sox2 being involved in maintaining the self-renewal properties of cancer stem-like cells suggests that these early embryonic transcription factors could be possible candidates for developing both treatment and prevention strategies against, which are aimed at specifically targeting this resistant population responsible for driving tumour formation.
Figure 1.9. Transcription regulatory network of pluripotency involving Oct4, Sox2 and Nanog. Pluripotency is individually regulated by Nanog, Oct4 and Sox2. Oct4 and Sox2 proteins bind together to form a complex which drives the expression of Nanog mRNA. Nanog protein binds to its own promoter region and also controls Oct4 transcription. Both Oct4 and Sox2 control their own transcription through feed-forward mechanisms. Also, when Oct4 is expressed above certain levels it downregulates both its own expression and that of Nanog.

1.15. Implications of the cancer stem cell hypothesis for cancer management

The interest in cancer stem-like cells from a clinical perspective arises from the inability of many treatment strategies to target this population, which may explain the therapeutic failures, cancer recurrence and metastases associated with current treatment regimens. Indeed, most cytotoxic chemotherapeutic drugs are effective in targeting the differentiated progeny in a tumour, whereas the slowly proliferating, cancer initiating or stem-like cell population are relatively resistant to such drugs. For instance, breast tumours that represent a basal phenotype, believed to be similar to the earliest mammary progenitor cells, are resistant to standard chemotherapy and patients have a
poor prognosis, which supports the notion that current therapeutic approaches fail to target cancer stem-like cells [153].

The cancer stem model, suggests that the development of new therapies targeting the stem-like compartment of cancers could potentially improve patient response. This model also provides significant inroads into the assessment of cancer risk and development of preventive strategies. As well as trying to target premalignant cancer stem-like cells it has been proposed that if normal stem cells or their immediate progeny are targets for transformation, then reducing their number in a particular tissue may reduce cancer risk. The use of tamoxifen in primary breast cancer prevention may occur through such a mechanism [66]. Expansion of the stem cell population by self-renewal represents an early event in carcinogenesis. As such, intervention strategies that selectively limit the self-renewal capacity of normal adult or premalignant stem cells by engaging apoptotic or differentiation pathways may be extremely valuable for cancer prevention. Additionally, such early interventions may inhibit the accumulation of mutational events in stem cells preventing the genetic alterations associated with neoplastic transformation.

Current methods for assessing the efficacy of chemotherapeutic agents in patients rely on measuring tumour volume/size. For a linear response, usually tumour shrinkage is taken into account for evaluating a clinically significant effect [66]. Currently there are set of guidelines called RECIST (Response Evaluation Criteria in Solid Tumour) that measures tumour size reduction (usually 30% reduction in the longest diameter of the tumour) as a measure of drug efficacy [154]. However, this may be misleading since cancer stem-like cells represent only a small fraction of the tumour mass and are essentially resistant to chemotherapeutic drugs. Hence, the shrinkage of tumour may only be representative of effects on differentiated progeny and not the cancer stem-like compartment. This could explain why in certain advanced cases, including many solid tumours and multiple myeloma, tumour regression does not correlate with a clinically significant enhancement of patient survival [155]. Accordingly, tumour shrinkage may not be the ideal parameter to measure the efficacy of drugs targeted against cancer stem-like cells. The development of antineoplastic agents based on the cancer stem cell
hypothesis therefore requires the identification of validated intermediate end points that predict the ultimate patient response to treatment regimens (Figure 1.10).

Figure 1.10. A comparative representation of cancer treatments. Conventional cancer treatments involve development of anti-cancer agents that essentially target the differentiated population of a tumour. The efficacy of such drugs is determined by their ability to shrink tumours at both the preclinical and clinical stages of their development. As the cancer or initiated stem-like cells are usually resistant to these drugs, they remain unaffected and may drive the recurrence of cancer at a later stage. Targeting the initiated or cancer stem-like cells that drive tumour progression using preventive or therapeutic agents could reduce cancer incidence and provide the possibility of a cure with time.
1.16. Aims and Objectives

Recent advancements in stem cell biology have given great impetus to the ‘cancer stem cell hypotheses’. Studies have shown that cancer stem cells, which constitute a small fraction of the tumour cell population, can be responsible for resistance against conventional cancer treatments, i.e. cytotoxic chemotherapy and radiotherapy. Curcumin, a constituent of the spice turmeric, has been suggested to interfere with the proliferation of cancer stem-like cells in colorectal cancer cell lines. However, there has been no information published addressing whether curcumin can target cancer stem-like cells in primary colorectal tissues. Therefore, this project aims to evaluate the potential efficacy of curcumin in terms of targeting cancer stem-like cells in patient-derived colorectal adenoma and cancer cells. A secondary aim is to investigate possible mechanisms of action through which curcumin might be able to inhibit the proliferation and expansion of cancer stem-like cells. In this respect, the embryonic stem cell transcription factor Nanog was identified as a potentially important molecular target, which led to the final goal of the project, aimed at unravelling how curcumin might target colorectal cancer stem-like cells via modulation of Nanog.

The major objectives of the project are as follows:

1) Identification and prioritisation of potential markers which might help guide anticancer efficacy studies of curcumin. To that end, human primary cells derived from normal, premalignant and tumour specimens were profiled for stem-like cell markers, i.e. ALDH activity and CD133 expression (Chapter 3).

2) Determination of whether curcumin targets the growth/proliferation of the stem-like compartment of primary colorectal tumours and adenomas in vitro (Chapter 4).

3) Exploring whether dietary curcumin can affect the development of cancer stem-like cell driven tumours in vivo using two murine colorectal cancer models, NOD/SCID mice bearing patient-derived colorectal tumours and Apc\textsuperscript{Min} mice which develop intestinal adenomas (Chapter 4).

4) Elucidation of the molecular mechanisms of action, though which curcumin targets colorectal cancer stem-like cells, with a particular emphasis on Nanog (Chapter 5).
Overall, this project was designed to assess the feasibility of using curcumin as a potential agent for the prevention and treatment of colorectal cancer via regulation of cancer stem-like cell proliferation and modulation of their behaviour. Ultimately, the results contribute new information which may also help judge whether the cancer stem cell models employed have a role to play in preclinical screening to aid candidate drug selection for clinical evaluation.
2. Chapter 2: Materials and Methods

2.1. Materials

All cell culture labware, unless otherwise stated, was ordered from Greiner, U.K or Appleton Woods, Birmingham, U.K. All stem cell media components were bought from Fisher Scientific except for Heparin which was purchased from Sigma. Cell strainers of 40 and 100 µm sizes were brought from VWR. All flow cytometer related products that included sheath fluid, CST (cytometer set up and tracking) beads, and Accudrop beads for drop delay set up were purchased from BD biosciences. For the Aldefluor assay, the Aldefluor Kit and Aldefluor buffer were brought from Stem cell technologies. Chemicals for general tissue culture that included trypsin, phosphate buffer saline and foetal calf serum, were purchased from Invitrogen. Industrial methylated spirit and xylene were purchased from Sigma.

Antibodies were purchased from different suppliers. CD133 (Cat no – 130-090-826) and EpCam (ESA, Cat no - 130-091-253) were brought from Miltenyi Biotech. ALDH1 (primary antibody – Cat no - 611194) was obtained from BD Pharmingen. DCAMKL-1 (Cat no - AP7219b) primary antibody and blocking peptide were ordered from Abgent. Secondary detection system (LSAB) and Avidin-Biotin blocking solutions used for IHC were obtained from Dako. Curcuminoinds (>94%) used for cell culture was purchased from Sigma. Meriva (20% curcuminoinds) and Epikuron used for the in vitro study were kindly supplied by Indena S.p.A., Italy. Resveratrol (99.9% purity) was supplied by Shanghai Novanet Co. Ltd., Shanghai, China.

2.2. Buffers

Water used for all buffers was double distilled.

2.2.1. Running Buffer (Western blotting)

Running buffer was made as a 1 in 10 dilution by diluting 100 mL of 0.25M Tris/1.92 M glycine/1 % SDS (10X) (Geneflow, UK) in 900 mL water.
2.2.2. Transfer Buffer
Transfer buffer was made as a 1:10 dilution by adding 100 mL of 0.25 M Tris/1.92 M glycine in 700 mL water and 200 mL methanol.

2.2.3. Ammonium persulphate (10%)
Ammonium Persulphate (AMPS) (Sigma, UK) was prepared by weighing 1g in 10 mL water (10%, w/v ratio). The stock solution was aliquoted and stored at -20°C for single use only.

2.2.4. Phosphate-buffered saline – tween-20 (PBST) (0.1%)
PBST was prepared by mixing 10 tablets of PBS (Sigma) and 1 mL tween-20 (Sigma) in 1000 mL of water.

2.2.5. Blocking buffer and antibody diluent (Western blotting)
For blocking purposes, 5% (2.5 g in 50 mL PBST) milk (Marvel, UK) was used. For diluting primary and secondary antibodies, 3% milk (1.5 g in 50 mL PBST) was used.

2.2.6. Zykovka buffer for pull-down assay
Zykovka buffer was made with 50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT) and 0.01% NP-40 (detergent). To make the complete buffer one tablet each of Phospho-stop (Invitrogen) and Complete Mini (Invitrogen) were added in 10mL of the Zykovka buffer.

2.2.7. Cell Lysis Buffer for Western Blots
All cell lysis including for both established cell lines and primary cells was done in RIPA buffer (Sigma). To make the complete lysis buffer one tablet each of Phospho-stop (Invitrogen) and Complete Mini (Invitrogen) were added in 10mL of the RIPA buffer.

2.2.8. Antigen retrieval buffer (Immunohistochemistry)
Citrate buffer was used for all antigen retrieval. Citrate (10 mM, citric acid) at pH 6.0 was freshly made up.
2.2.9. Antibody diluent (Immunohistochemistry)

Both primary and secondary antibodies were made in 1% (w/v) bovine serum albumin (BSA) in PBS. BSA (50 mg) was dissolved in 5 mL water. The antibody diluent was always freshly prepared prior to use.

2.2.10. Freezing mix

The freezing solution for storing single cells consisted of 10% dimethyl sulfoxide (DMSO) and 90% foetal calf serum.

2.2.11. Stem cell media composition

Stem cell media for both primary and established cells was prepared on a regular basis at a volume of 50 mL. The media could be stored at 2-8°C. The sphere media composition was as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume to add from Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Neurobasal Medium</td>
<td>25 mL</td>
</tr>
<tr>
<td>1% N-2 Supplement</td>
<td>500 µL</td>
</tr>
<tr>
<td>2% B-27 Supplement</td>
<td>1 mL</td>
</tr>
<tr>
<td>2% Antibiotic-Antimycotic</td>
<td>1 mL</td>
</tr>
<tr>
<td>2 µg/mL Heparin</td>
<td>50 µL</td>
</tr>
<tr>
<td>20 ng/mL FGF-2</td>
<td>10 µL</td>
</tr>
<tr>
<td>20 ng/mL EGF</td>
<td>10 µL</td>
</tr>
<tr>
<td>DMEM/F12 Medium (1:1) hyyclone</td>
<td>22.5 mL</td>
</tr>
<tr>
<td><strong>TOTAL 50 ml</strong></td>
<td></td>
</tr>
</tbody>
</table>
2.3. Cell lines

Both cell lines (Caco2 and HEK293) were purchased new for this project from ATCC (Middlesex, UK). Primary cells were isolated from colorectal cancer or adenoma tissue obtained from patients (see Section 2.4.2).

2.3.1. Maintenance and passaging of cell lines

Caco2 cells were originally derived from a colorectal adenocarcinoma resected from a 72 year old male and are representative of a moderately well differentiated colorectal cancer cell line. HEK-293 cells were originally derived from human embryonic kidney.

Caco2 and HEK-293 cells were cultured in Minimal Essential Medium (MEM, Sigma, UK) and high glucose (4500 mg/mL) Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 20% foetal calf serum (FCS) and 1X Glutamax (GIBCO, UK), respectively. Both cell lines were maintained in an incubator at 37°C supplemented with 5% CO₂ in a humidified atmosphere. Before harvesting, both cell types were grown to 70–80% confluency. Media was aspirated and cells washed with sterile PBS prior to addition of 1-2X trypsin/EDTA (T/E) for 5-10 min at 37°C. Following cell detachment, medium containing 10% FCS was added to neutralise the trypsin. Cell suspensions were then centrifuged at 1300 RPM (207 × g) for 3 min, the supernatant discarded and cells plated into new flasks at the required density, in complete growth medium. Cells did not exceed passage 20.

2.3.2. Sphere growth and maintenance

For growing spheres, freshly prepared single cell suspensions from Caco2 cells were plated at 20,000 cells per well in 6-well plates. Sphere growth was facilitated using sphere growth medium (2 mL/6 well), and by plating onto ultra low-attachment plates (Corning) or 6-well plates coated with 2 mL of 1% agarose in PBS. Sphere formation required a period of two to three weeks. Once spheres were established, they were passaged every 1-2 weeks and maintained by adding 500 μL of fresh sphere medium every 7 days. For passaging spheres, they were first filtered through 100 μm filters to remove any debris and the supernatant discarded. The filter was then inverted, and the
spheres gently washed through using DMEM supplemented with 10% FCS. Spheres were pelleted (1000 RPM, 123 × g, 5 min) and the supernatant removed. Trypsin (2X, 1 mL) was added to the cell pellets and incubated for 10 min at 37°C to dissociate the spheres, aided by gentle pipetting. Trypsin was neutralised with DMEM containing 10% FCS, and the cells pelleted (1000 RPM, 123 × g for 5 min) prior to re-suspending in 10 mL complete medium. Cells were counted using a Coulter counter (Beckman Coulter, UK). Cells were seeded at a density of 25,000 cells/well for FACS analysis and 10-30,000/well in sphere medium to enable study of the effects of curcumin on sphere growth. For sphere growth in 96-well plates, cells were seeded at 5-10 cells per well.

2.4. Analysis of human tissue samples.
Tissues were obtained as part of the ethically approved study ‘Development and application of stem cell assays for prevention and treatment of cancer’ (UHL 10741). Ethical approval was granted by Leicestershire, Northamptonshire and Rutland ethics committee (REC reference 09/H0402/45).

2.4.1. Logistics of tissue collection
All tissues collected remained fully anonymised to laboratory research staff. The samples consisted of resected diseased or normal tissues, and arrived at the laboratory by one of two routes: The first route was used for colon cancer or adenoma samples and the second route was used for colorectal liver metastasis samples.

1) The sample was collected from theatre and immediately transported (on ice) to the Pathology department at Leicester Royal Infirmary. The sample was then processed by a consultant pathologist (deputised by registrar if consultant not available). The pathologist only gave tissue in excess of that needed for diagnostic purposes. Two tissue specimens were required: fresh tissue that was placed directly into media 199 on ice, and tissue for formalin fixation. Tissue for formalin fixation was taken to the University-based pathology laboratory for processing. An internal patient ID number was generated to ensure patient anonymity.

2) Dissected tissue for research purposes was collected directly from theatre. One sample of tissue was placed directly into media 199 on ice, and another adjacent sample was taken for formalin fixation. The formalin fixed tissues were sent
directly to the appropriate pathology laboratory for processing. Patient anonymity was maintained throughout tissue procurement.

### 2.4.2. Tissue Processing

Diseased or normal tissues were collected from the operating theatre as described above. The fresh tissue mass was dipped into a bleaching solution for a few seconds (308 µL of 13% sodium hypochlorite in 250 mL water) to eliminate any bacterial or fungal contamination. The bleaching step was performed only for colon samples as there is greater potential for faecal contamination. The tissues were put into 5 mL of media 199 and cut into pieces with sterile scissors, until the suspension could easily be pipetted through a 10 mL pipette. After mincing was complete, the volume of media 199 was made up to 10 mL and the required amount of collagenase type 4 (Worthington Chemicals) added, to obtain a working concentration of 2000 U/mL. The tissue suspension was then incubated at 37°C in a rotatory mixer for 60-90 min (depending on tissue type), during which time cells were pipetted every 15 min. Cells were examined on slides every 30 min to determine if further digestion was required. Following collagenase digestion, the cell suspension was filtered through a 100 µm filter (BD Falcon) to discard any large lumps of undigested tissues. The cell suspension was then centrifuged at 350 × g for 5 min, the supernatant removed and cells washed with HBSS (Sigma) for 5 min. The cell suspension was filtered again through a 100 µm filter and washed twice with HBSS. After the final wash, the sample was passed through a 40 µm filter and the filtrate, which contained a single cell suspension, was collected. These cells were suspended in freezing mix and immediately frozen, used for FACS analysis, or seeded into ultra-low attachment plates for sphere growth.

### 2.5. Staining of primary and established cell lines for ALDH activity

The Aldefluor™ assay kit (Stem Cell Technologies, UK) provided all the reagents required to perform the assay. The inactivated Aldefluor substrate was first dissolved and activated according to the manufacturer’s instruction. In brief, 25 µL of DMSO was added and mixed with the dry Aldefluor substrate (Biodipy™ – aminoacetaldehyde) for 1 min. Following which, 25 µL of 2N hydrochloric acid was added and 15 min incubation performed at room temperature. The solution was then mixed with 360 µL of Aldefluor assay buffer and the activated substrate aliquoted and stored at -20°C.
All reagents, including the activated Aldefluor substrate were allowed to reach room temperature before use. The minimum number of cells required to perform the assay was 50,000 with an upper limit of $2 \times 10^6$ cells. After harvesting cells or preparing single cell suspension, cells were re-suspended in 1 mL of Aldefluor buffer. For each sample to be tested for ALDH activity, an ALDH inhibitor, diethylaminobenzaldehyde (DEAB) was used as a negative control. Briefly, 10 µL DEAB was added to 500 µL of cell suspension and immediately transferred to an eppendorf containing 2.5 µL of activated Aldefluor substrate and mixed. For the samples to be tested for ALDH activity, activated 5µL aldefluor substrate was mixed with 1mL of cell suspension. Following this, all samples were incubated at 37°C for 40 min. The activated substrate was converted by intracellular (ALDH) into a negative charged fluorescent compound (BIODIPY™-aminoacetate). The negative charge of this reaction product prevents diffusion and retains it inside the cells. However this can be easily effluxed from cells by the ATP-binding cassette (ABC) transporter system. This active efflux is prevented by the use Aldefluor assay buffer that contains inhibitors of the ABC transporter system. After the incubation period, all cell samples were washed with 500 µL of Aldefluor buffer and centrifuged at 207 × g for 3 min. The supernatant was discarded and the pellet was re-suspended in 500 µL of Aldefluor buffer. The cell suspension was then transferred to a FACS tube for analysis using a flow cytometer. For dual or triple staining with other antibodies, their respective staining protocols were used following the Aldefluor assay.
Figure 2.1 A pictorial representation of the Aldeflour assay. Inactivated substrate (BAAA-AA) is converted into its active form by acid treatment. The activated substrate (BAAA) is fluorescent and nontoxic that can freely diffuse into live cells where it is converted by aldehyde dehydrogenase (ALDH) into BAA-. The reaction is performed in Aldefluor buffer that contains inhibitor of the ATP-binding cases (ABC) system, thus preventing the active efflux of the reaction product and retaining it inside the cells. The resulting fluorescence is then measured by a flow cytometer. A specific ALDH inhibitor (DEAB) is used as a negative control.

2.6. Staining for CD133 and ESA

The following protocol is appropriate for single or multiple staining using antibodies against CD133 and ESA. However, when used in conjunction with the Aldeflour assay, Aldeflour assay buffer was used at all times to avoid loss of the Aldeflour signal. Staining was performed on a single cell suspension of either freshly harvested cells or cells stained for ALDH activity. For all samples, the single cell suspension was washed with 500 µL of Aldeflour assay buffer, centrifuged at 207 × g for 3 min and the pellet resuspended in 100 µL of Aldeflour assay buffer. Following this, single, dual or triple staining could be carried out by addition of CD133 and/or ESA antibodies at a dilution of 1:10 in the 100 µL cell suspension. The cells were incubated with the antibodies (30 min, 4°C in the dark) then washed and re-suspended in Aldeflour assay buffer (500 µL) and the resulting cell suspension analysed via flow cytometry.
2.7. **FACS analysis and sorting conditions**

All FACS analysis and sorting was performed using a Becton Dickinson BDFACS Aria II special order research product (SORP). The instrument was subjected to performance checks prior to any sample analysis. For sorting cells, a laser delay was set up using Accudrop beads (BD Biosciences) each time a sort was performed. For maintaining aseptic condition, sample lines were cleaned with FACS clean (BD Biosciences) followed by a rinse with sterile PBS. The nozzle used for sorting or analysis of cells was 85 µm in size. The flow chamber, deflection plates and sample loading area were all cleaned with 70% IMS prior to sorting. For triple colour experiments, appropriate compensation was set up and applied to the sorting experiment to avoid any crossover of fluorescence signals from respective fluorochromes. The sorted cells were collected in a tube holder consisting of 4 tubes filled with sample collection medium (DMEM media supplemented with 10% FCS). The sorting gates were set up to exclude any doublets. The minimum number of events recorded for any analysis was 10,000, provided that there were enough cells for analysis.

2.8. **Sphere forming assays**

2.8.1. **Primary cell treatments**

Cells from either primary tissues or established cell lines were used for sphere forming assays. For sphere formation, a single cell suspension was used. Primary single cells were plated at a density of 30,000 cells/well in six-well ultra-low attachment plate in sphere media and left O/N (10% CO₂, 37°C) prior to treatment. Primary spheres were grown for 4 weeks with or without treatment. For treatments, curcumin or resveratrol in DMSO was added to achieve final concentrations of 0.01, 0.1, 1, and 5 µM twice weekly for a period of 4 weeks. When adding curcumin or resveratrol, the appropriate volume (100 µL) was added on top of the existing incubation volume at the time of treatment. This was done to avoid any centrifugation step that could potentially hamper or disrupt sphere growth. Sphere numbers and size were measured following 4 weeks of growth as described below. All treated and control incubations contained an equivalent concentration of DMSO (diluted in media), which did not exceed 0.1%.
Following treatment, spheres were harvested for subsequent counting and size measurement. All treatments were done in triplicate, typically on three separate occasions, for each patient sample. Spheres from each well were collected in a 15 mL centrifuge tube and spun at 191 × g for 10 min. The supernatant was discarded leaving a residual volume of 30-50 µL in the centrifuge tube with the sphere pellet. Gridded slides were circled around the grid with wax to define an area within which the spheres were counted and measured. Sphere pellets were re-suspended in the residual volume and plated into the circled area of the gridded slides. Maximum care was taken to avoid any bubbles while re-suspending the sphere pellet as this would affect sphere counting. Cover slips were placed gently on top of the sphere suspension in the gridded slides making a seal with the waxed circle. This was done to hold the spheres in place for counting and measurement and also to prevent any leakage and drying out of the sphere suspension. Following slide preparation spheres were counted and their size measured using an inverted light microscope (Nikon EclipseTE2000U) at 10X optical zoom. Sphere size was determined using Eclispe software that measured an average diameter of two length measurements across each sphere. For all treatment studies, samples were coded by an independent person and sphere analysis was undertaken blind to avoid potential bias.

2.8.2. Flow cytometry

Following treatment, single cell suspensions were obtained from spheres for FACS analysis. Briefly, spheres were centrifuged (191 × g, 10 min), the supernatant discarded and 2 mL trypsin/EDTA added at 5X concentration to each sphere pellet, which was then incubated at 37°C for 10 min. Following digestion, 4 mL media containing fetal calf serum (10%) was added to neutralise the trypsin. At this stage, samples were mixed vigorously using a 1mL pipette to ensure there were no visible cell aggregates remaining. Cells were washed in PBS and centrifuged at 207 × g for 3 min. For FACS analysis cells were re-suspended in Aldefluor buffer for staining with CD133 and ESA antibodies and assessment of ALDH activity (see Sections 2.5 and 2.6). All FACS analysis was undertaken using a BD FACS Aria II with Diva software 6. For each concentration, Aldefluor assays included a respective control and test specimen. For CD133 and ESA, unstained cells were used as controls for FACS analysis. For triple
staining (ALDH, CD133 and ESA), the Aldefluor assay was performed prior to CD133 and ESA staining.

2.9. **In vivo studies in NOD/SCID mice**

2.9.1. **Propagation of primary tissues or cells in NOD/SCID mice**

Male NOD/SCID (NOD/SCID NOD.CB17/JHlsiHsd-Prkdcscid) mice were obtained from Harlan laboratories, UK, at an age of 5-6 weeks. All mice were fed on normal irradiated diet (5LF-5) for maintenance. The animal work was performed under project licence number - 60/4370 awarded to the University of Leicester.

For tumour implantation, patient-derived colon tumours from surgical resections were carefully sliced into small pieces (about 2mm thick) and put into media 199. Before implantation, the tumour specimens were washed twice in media 199 containing 2% Antibiotic-Antimycotic (Invitrogen). Prior to implantation, mice were anaesthetized with a controlled exposure to isoflurane gas. Once anaesthetized, mice were shaved on the lower back region, into which the tumour pieces would be engrafted. A small pocket was made in the right and left flank of the animals, into which two small pieces of tumours were placed. Lidocaine (2mg/kg) was used as a local anaesthetic prior to suturing. For studies involving single cell injection, cells were mixed with matrigel in a 1:1 ratio by volume and injected subcutaneously (100 μL) into the right and left mid-abdominal areas using a 25 ½ - gauge needle.

Body weight and tumour size were measured twice a week. After tumours reached an estimated size equivalent to 5% of the animal’s body weight (approximately 17 mm diameter) or earlier if the tumour ulcerated, animals were sacrificed by cardiac puncture under terminal anaesthesia and the tumour excised.

Tumour tissues obtained from engraftment in NOD/SCID mice were routinely handled in the following manner: A portion was processed into a single cell suspension and a proportion of these cells was used for performing various stem cell assays and the rest frozen down and stored in liquid nitrogen for further use; a small portion of the tissue was fixed in formalin for histopathology; the remaining tissue was used for further
serial transplantation in NOD/SCID mice to generate serial xenografts following the same procedure as above. Serial transplantation in to NOD/SCID mice was performed on a regular basis to maintain a steady supply of primary tumours in mice for use in subsequent experiments.

2.9.2. Injection of sorted cells into NOD/SCID mice
Primary human cancer cells obtained from tumours passaged in mice were used for FACS based sorting and subsequent injection into NOD/SCID mice. Cells obtained from serially implanted tumours (usually at passage 2-4), were stained for ALDH activity using the Aldefluor assay kit, as described in Section 2.5, sorted under sterile conditions and plated into stem cell media in ultra-low attachment plates (10% CO₂, 37°C) for a period of 48 h. This was done to allow the cells to recover from the stress of cell sorting. Usually for injection, twice the number of cells required was sorted to account for approximately 50% death due to sorting. Following cell recovery, a trypan blue assay was performed to assess the viability of the cells. Viable sorted cells were then injected into the right flank of NOD/SCID mice according to the method described in Section 2.9.1.

2.9.3. In vivo efficacy Studies
For curcumin efficacy studies, NOD/SCID mice were placed on 5-LF5 irradiated powdered diet containing either 0.9% Epikuron (n=5), or 1.13% Meriva (n=5). One week after commencing these diets all mice, were injected with 2000 ALDH<sub>high</sub> colorectal cancer cells into the right flank. These cells were obtained from a serially implanted human tumour and were all from the same patient. Following implantation, animals were maintained on their respective diet for the rest of the study period.

Tumour formation was monitored for a period of 16-20 weeks. Body weight was recorded once a week and tumour size measured at least once a week, once tumour growth was established. After tumours reached an estimated size equivalent to 5% of the animal’s body weight (~17 mm diameter), or earlier, if the tumour ulcerated, animals were sacrificed by cardiac puncture under terminal anaesthesia.
2.10. DCAMKL-1 Staining in \textit{Apc}^{\text{Min}} \textit{mice}

2.10.1. DCAMKL-1 staining over time

Female \textit{Apc}^{\text{Min}} mice were randomized at weaning into 4 different groups (n=4 per group) and were culled at different ages to assess DCAMKL-1 staining in the small intestine at various stages of development. At 5, 8, 12 or 16 weeks of age, mice were euthanized by exsanguination (normally with thoracotomy) whilst under terminal gaseous anaesthesia. The alimentary canal was then flushed through with PBS and removed. Intestines were collected into swiss rolls and fixed in 10% buffered formalin then paraffin embedded for subsequent DCAMKL-1 immuno-staining in different regions of the intestine.

2.10.2. Effect of curcumin on intestinal stem-like cells in \textit{Apc}^{\text{Min}} \textit{mice}

To assess the effects of curcumin on the number and location of intestinal stem-like cells in \textit{Apc}^{\text{Min}} mice archived formalin fixed paraffin embedded tissues from a previously published study [31] were immunohistochemically stained for DCAMKL-1. Briefly, male \textit{Apc}^{\text{Min}} mice were randomised into control and treatment groups that received 0.1%, 0.2% and 0.5% dietary curcumin. At the end of treatment (18 weeks), all mice were culled and the intestine was collected as described in Section 2.10.1. [31].

To assess the effects of curcumin on normal intestinal stem cells, wild-type male C57/BL6 mice were maintained on either control diet or a diet containing 0.5% curcumin. At the end of the experiment when mice reached 18 weeks, they were culled by cardiac exsanguination under terminal anaesthesia. The intestine was collected and fixed in formalin as described in Section 2.10.1.

2.10.3. Immunohistochemistry for DCAMKL-1 expression in intestinal tissue of \textit{Apc}^{\text{Min}} \textit{mice}

Paraffin-embedded sections were dewaxed by heating (30 min, 70°C) then slides were hydrated in a sequence of 3 min washes starting with xylene to graded alcohol (99% to 95% industrial methylated spirit). Antigen retrieval was performed by microwaving in citrate buffer (pH 6) for 18 min on high power (corresponding to 99°C). Endogenous biotin activity was blocked using an Avidin/Biotin blocking kit (Dako) according to the
manufacturer’s instruction. Slides were then washed twice for 5 min in PBS and incubated for 20-30 min in 3% hydrogen peroxide (Sigma, UK) to eliminate endogenous peroxidase activity. Slides were washed in PBS (5 min) and incubated with 2% goat serum at room temperature (RT) for 20 min to block non-specific binding. The sections were then exposed to primary rabbit polyclonal anti DCAMKL-1 at a dilution of 1:1000, overnight at 2-8°C. As a negative control, the primary antibody was blocked using a blocking peptide (Abgent) targeted against DCAMKL-1 epitope, according to the manufacturer’s instruction. Following primary antibody incubation, slides were washed twice in PBS (5 min) and incubated in the appropriate secondary biotinylated donkey anti-rabbit antibody (Jacksons Immunoresearch, 30 min, RT). Slides were washed again in PBS and incubated in streptavidin horseradish peroxidase (Dako) for 12 min at RT. After a final wash in PBS, slides were incubated (10 min, RT) in DAB substrate (Dako) to allow chromogenic development. Slides were then rinsed in water and counter-stained with haematoxylin (30 sec), following which, slides were rinsed again in water, dehydrated in graded alcohol, cleared in xylene and mounted permanently with DPX mounting solution.

2.11. **Ki-67 staining in Caco2 cells**

2.11.1. **Cell sorting and treatment**
Caco2 cells were seeded at a density of 0.4x10⁶ per 175 cm² large flask and treated with curcumin at 0.1 and 1 µM, along with a DMSO solvent control. To model the repeat treatment schedule adopted in the primary sphere studies described above (section 2.8.1), fresh curcumin was added three times over a 7 day incubation period. For each treatment concentration, a total of 9 large flasks were required. Cells were harvested a day after the last treatment and stained for ALDH activity using the Aldefluor assay kit. Following ALDH staining, Caco2 cells were sorted into ALDH⁺high and ALDH⁺low populations for each treatment. A total yield of 0.5-1 million ALDH⁺high/low cells were obtained following FACS sorting.

2.11.2. **Ki-67 staining**
Sorted Caco2 cells (ALDH⁺high/low) were first fixed in methanol for 10 min at -20°C and permeabilised with 0.5% tween-20 or triton-X in PBS. Following fixation and
permeabilisation, cells were incubated in mouse polyclonal anti-Ki-67 antibody at a dilution of 1:10 (40 min, in the dark, 4°C). All antibody dilutions and washes were performed using 3% BSA in PBS. After primary antibody incubation, cells were washed and incubated (30 min, in the dark, 4°C) in secondary anti-mouse conjugated Alexa Fluor@488 antibody then washed twice. For negative controls, single staining with secondary antibody was performed to eliminate any background staining. Cells were analysed for Ki-67 staining using the BD FACS Aria II.

2.12. Proteome profiler

The Pluripotent Stem Cell proteome profiler kit (R&D systems) was used to assess expression of a panel of stem cell-related proteins in cells obtained from patient-derived spheres. The dosing strategy used was identical to the one that was used for primary sphere treatment (see Section 2.8). After treatment, spheres were harvested according to the method described in Section 2.8.1. Once a single cell suspension was obtained, cells were lysed in lysis buffer 16 (provided in the proteome profiler Kit). The lysate was centrifuged (14,000 × g, 10 min, 4°C) then the supernatant was collected and stored on ice for protein quantification (see Section 2.13.2).

All reagents were prepared according to the manufacturer’s instruction. Briefly, each array (the nitrocellulose membrane containing the antibody array) was blocked with 1mL array buffer provided in the kit for 1 h at RT on a rocking platform. The cell lysate was diluted to a concentration of 50 µg protein in a final volume of 1mL lysis buffer. Following blocking, the array was incubated with the protein lysates at 2-8°C O/N, on a rocking platform. A detection antibody cocktail was diluted as per the manufacturer’s instructions. Following overnight incubation the array was washed twice with wash buffer then incubated (RT, 2 h) with the detection antibody cocktail on a rocking platform. The detection system used a Biotin labelled secondary antibody. Hence, a further incubation was performed with streptavidin–HRP complex (30 min, RT) and the array was again washed twice in wash buffer, before detection by chemiluminescence on an X-ray film (see Section 2.13.6 for details).

2.13. Western blotting analysis of Caco2 cell lysates

For cell treatment and sorting see Section 2.11.
2.13.1. Production of cell lysates

After treatment, cells were harvested according to the method described in Section 2.1.1. For phosphorylated proteins, cells were harvested and immediately transferred to ice. Ice cold PBS was used to wash the cells, which were kept on ice throughout processing. RIPA buffer (Sigma, UK) was added to the cell pellets in a 1:2 ratio (w/v). The lysis buffer was supplemented with 1 tablet of Phospho-Stop (Roche, UK) and Complete Mini (Roche, UK) per 10 mL RIPA buffer to make a complete lysis buffer. Cells in the complete lysis buffer were kept on ice for 30 min, following which they were frozen at -80°C O/N. The cell lysate was defrosted and centrifuged (207× g, 10 min), the supernatant was collected and the protein concentration determined using the Bradford Assay.

2.13.2. Bradford Assay

Bio-rad reagent was diluted 1:5 in water. A total of 1 mL diluted Bio-rad reagent was used for each protein concentration that was measured. The protein was diluted 1:1000 or 1:200 depending on the protein yield, in 1 mL Bio-rad reagent and the absorbance was measured at 595nm using a spectrophotometer. Water was used as the reference solvent along with the Bio-rad reagent (without any protein) as a blank, which was subtracted from the absorbance value determined for each protein sample. Protein concentration was calculated using a calibration standard curve that was generated by measuring the absorbance of known concentrations of bovine serum albumin (1, 2, 4, 6, 8, 10, 12, 15, 20 and 25 µg/mL).

2.13.3. SDS – PAGE

For all proteins investigated a 10% gel was used, since the molecular weights of the proteins ranged between 35-43 kDa. A gel casting apparatus (Bio-rad, mini gel apparatus) was set up according to the manufacturer’s instructions. Stacking and resolving gels were prepared at 5 and 10%, respectively (Table 2.1). The resolving gel was made first, poured into a gel cassette and allowed to set, then the stacking buffer was poured on top of the resolving gel and a 10 or 15 well comb inserted into the stacking gel. Once set, the comb was removed and the gel cassette placed in the running tank with 1X running buffer.
2.13.4. Running and transferring protein samples

Protein samples were defrosted on ice. Appropriate volumes of protein lysate were diluted in water to provide samples of equivalent concentration in a final volume of 10 µL. An equal volume (10 µL) of Sample loading buffer (2X Laemmli, Sigma, UK) was added to the protein lysate, making a total loading volume of 20 µL. Samples were then heated (5 min, 100°C) to denature the proteins, followed by a quick vortex and a pulse centrifuge. Samples were subsequently loaded into the wells and run at 40 mV for 60-90 min at RT.

Proteins were transferred from the gel on to a nitro cellulose membrane as follows: Gels were placed into a transfer cassette, which consisted of a sponge, blotting paper (Whatmann, UK), the gel, the nitrocellulose membrane (Geneflow Ltd UK), blotting paper and a final sponge. The transfer cassette was placed into a mini gel holder with colour coding that ensured the correct orientation of the transfer cassette. All blotting paper, nitrocellulose membrane, and sponges were pre-soaked in 1 X transfer buffer before being placed in the transfer cassette. The transfer cassette was then inserted into a blotter assembly in a tank filled with transfer buffer. Protein transfer was undertaken at 100 V for 2 h at RT.

2.13.5. Blocking and Antibody probe

Following transfer of protein into the nitrocellulose membrane, the membrane was blocked in 5% milk (in PBS with 0.1% tween 20), at RT for 2 h. The membrane was then washed in PBST for 10 min, followed by a further 2 X 5 min washes. The primary antibody diluent was prepared in 3% milk in PBST at the required dilution (see Table 2.2 for antibody dilutions). The membrane was incubated with the primary antibody O/N at 4°C on a rocking platform, before washing for 10 min, followed by two further 5 min washes in PBST. The membrane was incubated with the secondary antibody, prepared in the same diluent as the primary, for 1 h at RT on a rocking platform. Finally, the membrane was washed twice in PBST (10 min, then 5 min) followed by water (5 min, RT).
2.13.6. Detection of proteins
After the final wash, the membrane was placed in an Enhanced Chemiluminescence Luminol (ECL) solution (Geneflow Ltd, UK) for 3 min at RT. The ECL reagent was prepared by mixing two reagents, A and B, (supplied by the manufacturer) in a 1:1 ratio for 5 min at RT. After 3 min exposure, the excess solution was drained off and the membrane wrapped in cling film and put into an X-ray developing hypercassette (Amersham, UK). In a dark room, the membrane was exposed to X-ray film (GE healthcare, UK) for 1-5 min and the film was developed using an Agfa Curix 60 developer (AGFA Gevaert N.V, Germany).

2.13.7. Verifying equal loading
To confirm that proteins were loaded in equal amounts, protein quantitation was normalised using a housekeeping protein. This was achieved by re-probing the membrane with actin (Santa Cruz, UK). Quantitation was performed by measuring the protein band intensity using GeneSnap version 6.01 (SynGene, UK) on a Bioimaging system (Syngene, UK). The software package used for quantitation included Gene Tools version 3.03.03 (Syngene, UK).

Table 2.1 Reagents for 5 and 10% gels

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<th>Reagents</th>
<th>5% gel (volume)</th>
<th>10% gel (volume)</th>
</tr>
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<tbody>
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<td>Distilled water</td>
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<td>ProtoGel, 30%(w/v), Acrylamide (Geneflow, UK)</td>
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<td>Protogel, Resolving or Stacking buffer (Geneflow, UK)</td>
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Table 2.2 Antibody dilutions

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<tr>
<th>Primary antibody</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog (Novus Biologicals)</td>
<td>1:1000</td>
<td>1:5000</td>
</tr>
<tr>
<td>Cat no - 29290002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oct4 (Novus Biologicals)</td>
<td>1:1000</td>
<td>1:5000</td>
</tr>
<tr>
<td>Cat no - NB100-2379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox2 (Millipore)</td>
<td>1:1000</td>
<td>1:5000</td>
</tr>
<tr>
<td>Cat no – ABS603</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-Nanog</td>
<td>1:1000</td>
<td>1:5000</td>
</tr>
<tr>
<td>Cat no - PA5-13078</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin (Santa Cruz)</td>
<td>1:1000</td>
<td>1:20000</td>
</tr>
<tr>
<td>Cat no – sc1616</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.14.  Real time qRT-PCR methods

2.14.1. RNA extraction and elimination of genomic DNA

Total RNA was isolated from FACS-sorted cell populations (see section 2.11.1) using an RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Pellets containing <5x10^6 cells were lysed in 350 µl of RLT buffer (provided in the Qiagen kit) before homogenising using the QIAshredder spin columns (Qiagen). The optional DNase digestion step was included to eliminate genomic DNA contamination; DNA was digested using the RNase free DNase set (Qiagen) following the manufacturer’s instructions. Total RNA was eluted into a final volume of 20 µL nuclease free water (Invitrogen) and stored at -80°C until further analysis.

2.14.2. RNA quantification

Total RNA was quantified using a NanoDrop spectrophotometer (Nanodrop Technologies). For each sample, a volume of 2 µL was analysed in duplicate and an average reading obtained. A 260/280 nm absorbance ratio of ~2.0 was an indicator of
pure RNA. The 260/230 nm ratio was taken as an additional measure of purity and was considered acceptable if within the range ~2.0-2.2. RNA samples were discarded if the absorbance did not fall within the above ranges.

2.14.3. Synthesis of first strand cDNA and further elimination of genomic DNA

cDNA was synthesised from extracted total RNA using the RT² First Strand Kit (Qiagen). Total RNA input into the reaction was 0.8 or 0.5 µg, depending on the experiment. The manufacturer’s instructions were followed, which included a genomic DNA elimination step before the reverse transcriptase reaction. The final cDNA reaction mixture was made up to a total volume of 110 µL with nuclease free water (Invitrogen) before storage at -20°C.

2.14.4. Real time qPCR

Real time PCR reactions were performed using an ABI StepOne Plus real time PCR machine. A TaqMan Fast Universal Master Mix (2x, Life Technologies) was added at 5 µL per reaction and target specific TaqMan FAM-MGB probe (20x, Life Technologies, see Table 2.3 below) were added at 0.5 µL per reaction, cDNA was added at 4.5 µL per reaction. Reactions were performed in MicroAmp Fast Optical 96-well PCR reaction plates (Applied Biosystems) with Optical Adhesive covers (Applied Biosystems). The standard short cycle parameters were used; 95°C 40 sec plus 40 cycles of 95°C 1 sec, 60°C 20 sec.

Table 2.3. Amplicon lengths of target genes. TaqMan probes were obtained from Invitrogen.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Amplicon length (base pairs)</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NANOG</td>
<td>121</td>
<td>HS04260366_g1</td>
</tr>
<tr>
<td>SOX2</td>
<td>91</td>
<td>HS01053049_s1</td>
</tr>
<tr>
<td>OCT4</td>
<td>64</td>
<td>HS00999634_gH</td>
</tr>
<tr>
<td>ACTIN</td>
<td>171</td>
<td>HS99999903_m1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>58</td>
<td>HS03929097_g1</td>
</tr>
</tbody>
</table>
2.15. Pull-down assays

Curcumin or resveratrol was immobilised onto agarose beads in two different orientations using a short amine linker prepared by Dr Robert Britton (Chemistry Department, UOL). Briefly, Curcumin functionalised beads were produced by covalently attaching curcumin to agarose beads by the reaction between a short amine linker (on the 4-OH position of curcumin) and the N-hydroxysuccinimide activated ester of Affigel-10. Resveratrol beads were made in a similar manner but 2 different attachment points were used, and control beads were produced by reacting Affigel-10 with ethanolamine (Figure 2.1). Optimised methods for the use of these beads in protein pull-down assays had already been developed in-house by Dr R. Britton and Dr E. Horner-Glister and were adopted in this study. The curcumin or resveratrol beads were incubated with cell lysate overnight at RT. The cells were lysed in Zykova buffer. The following day, the beads were washed several times with Zykova lysis buffer to remove unbound proteins, and curcumin or resveratrol-bound proteins were eluted from beads by heat denaturing in SDS buffer for 5 min at 95° (Sigma, UK). The pull-down was conducted in parallel with control beads end capped with ethanolamine and 500 µg of total cellular protein was used in each pull. For recombinant Nanog (Sigma, UK), 0.5 and 1 ng protein was used for the pull-down. The eluted proteins were separated by PAGE followed by immunoblotting to verify the presence of known targets (see Western blotting, Section 2.13).
Figure 2.2. Beads used for pull-down assays. Beads were synthesised in house by Dr Rob Britton. The control beads were the same for both curcumin and resveratrol pull-downs. For the resveratrol pull-down experiments a 1:1 mixture of 4´-resveratrol and 3-resveratrol beads were used.

2.16. Cycloheximide assay for monitoring protein turn-over
A cycloheximide (CHX) (Sigma, UK) assay was conducted using Caco2 and HEK293 cell lines to investigate the effects of curcumin on Nanog protein turnover. Cells were seeded in a medium flask (75cm²) at a density of 1x10⁶ per flask, a day before conducting the CHX assay. On the day of the experiment, cells were exposed to 100 μg/mL of CHX for 0.25, 0.5, 1, 2, 4, and 8 h and were then harvested and lysed to enable Nanog expression to be investigated by Western blotting. For curcumin treatments, cells were pre-exposed for 1 h to 0.1 μM curcumin prior to the addition of CHX. Cyclin D1 (Cell Signalling, UK) expression was used as a positive control for the CHX assay. All data were normalised to Actin expression. For details of Western blotting see Section 2.13.
2.17. **Tryptophan fluorescence assay**

The intrinsic fluorescence of tryptophan residues in Nanog was measured for the binding assay. The experiment was conducted with full length recombinant Nanog (Sigma, UK) and fluorescence was measured using a Fluoromax 4 Spectrophotometer (Horiba). The recombinant protein was reconstituted in binding buffer (0.2% tween20 in PBS) to a 4 µM stock concentration and for the binding reaction this stock was further diluted to 40 nM with the same buffer. Curcumin was serially diluted from a stock solution of 50 mM to 1000, 500, 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 nM working concentrations in the binding buffer, as mentioned above. At each concentration, the protein and curcumin mixture was incubated for 2 min at RT to allow binding to occur. The protein and curcumin mixture was transferred to a quartz cuvette and the tryptophan fluorescence was measured on the spectrophotometer. The excitation and emission wavelengths used were 295 nm and 310-500 nm, respectively.

2.18. **Circular Dichroism Spectroscopy**

Wavelength spectra using circular dichroism (CD) were collected for the recombinant full length Nanog protein with or without curcumin over a range of temperatures to construct a thermal denaturation curve. The protein was reconstituted in CD buffer, consisting of 10 mM Na₂HPO₄, 137 mM NaF, and 1.8 mM KH₂PO₄ at pH 7.4, to a working concentration of 75 ng/µL. The thermal denaturation curve was generated by a temperature ramping experiment. The protein itself in the presence or absence of 0.1 µM curcumin was heated from 20°C to 94°C at 1°C per minute heating rate and the wavelength spectrum was collected every 2 °C from 260-190 nm with 1 nm steps. The thermal curves were analysed by global3 software. The CD wavelength spectra were measured on a Chirascan™-plus CD Spectrometer (Applied Photophysics).

2.19. **Statistical analysis**

For statistical analysis, Student T-test was used, with the null hypothesis that any p-value below 0.05 represents a statistically significant difference between two parameters. An F-test was performed to check for equal variance of data. For regression analysis, Pearson correlation coefficient was used to evaluate any correlation between two data sets.
3. Chapter 3

3.1. Introduction

The cancer stem cell hypothesis has revealed a new paradigm of cancer research aimed at developing prevention or therapeutic approaches through targeting cancer stem-like cells. It has become increasingly evident that cancer stem-like cells do exist and play a crucial role in the process of carcinogenesis. As such, the characterisation of this population in the context of carcinogenesis is an important aspect towards understanding the biology of these cells. Various groups have used cell-specific markers to isolate and characterise cancer stem-like cells in different tumour systems. For example, in colon cancer it has been shown that tumour initiating stem-like cells are characterised by increased expression of a cell surface antigen, CD133 [78]. Huang et al. have also documented the use of aldehyde dehydrogenase 1 (ALDH1) activity as a marker of stemness in both premalignant and malignant primary colon [89].

Both in vitro and in vivo methods have been used to evaluate the tumourigenicity of cancer stem-like cells. Sphere forming assays have been increasingly used as a model to assess the stem-like properties of cells derived from primary cancers [78]. In this system stem-like cells exhibit growth in aggregate form resembling spheroid-like structures in serum free medium supplemented with growth factors. Both normal and neoplastic stem-like cells from neural and epithelial organs can be expanded as spheroid aggregates [123, 156, 157]. These spheroids harbour the cancer stem-like population in an undifferentiated state and are also representative of the cancer stem-like cell marker profile of the primary tumour. As such, in cultures derived from primary colon cancer tissue, spheres consistently contained a CD133 expressing population [78]. In vivo systems used include NOD/SCID mice to assess and characterise the properties of these cancer stem-like cells. The principle behind this model is that injection of low numbers of cancer stem-like cells, isolated based on the expression of certain markers, into mice results in tumour formation, whereas injection of cells with a negative phenotype yields no tumour formation. This has been demonstrated for primary colon cancer, where subcutaneous injection of 3000 CD133+ cells showed tumour growth within 3-4 weeks in NOD/SCID mice, but injection of a much higher number (10^5) of CD133− cells failed to generate tumours, indicating the stem-like population resides within the CD133+.
fraction [78]. Similar data exist for ALDH activity in colon and breast cancer, where injection of ALDH$^{\text{high}}$ cells shows tumour formation, whereas the ALDH$^{\text{low}}$ cells produced no tumours in NOD/SCID mice [89]. Serial passaging of these tumours in NOD/SCID mice resulted in tumours that were morphologically similar to the original tumour, which suggests that the cancer stem-like cells could self-renew \textit{in vivo}.

In human colorectal cancer, CD133 expression and ALDH activity have mainly been used as markers for the isolation and \textit{ex-vivo} functional characterisation of the cancer stem-like population. However, when this project was started there were no extensive studies published which investigated the relevance of CD133 and ALDH as markers of cancer stem-like cells in the carcinogenic progression of colorectal cancer. Therefore, the aim of the work described in this chapter was to identify the most appropriate cancer stem-like markers, based on their presence in primary cancer and adenoma tissues, by evaluating their specificities and examining whether patient characteristics influence the presence and size of particular cancer stem-like populations. Selection of such markers was necessary before they could be used to help evaluate the effects of cancer preventive/therapeutic agents on the stem-like fraction in subsequent experiments. This chapter also aims to establish both \textit{in vitro} and \textit{in vivo} methods of propagating cancer stem-like cells. Hence, this section describes how the profile of these markers changes with disease progression in normal, adenoma and cancer tissue, and across different pathological stages in primary colorectal cancer. Both \textit{ex vivo} sphere assays and patient-derived xenografts in NOD/SCID mice were used to assess the tumourigenicity and growth characteristics of primary colorectal tumours obtained at surgical resection, with respect to the original stem-like population. Changes in ALDH activity and CD133 expression were also monitored following \textit{in vitro} propagation and serial passaging \textit{in vivo}. 

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3.2. **Comparison of ALDH activity and expression in primary colorectal tumours**

The Aldefluor assay, as outlined in the Methods Section, is based on the activity of the aldehyde dehydrogenase enzyme; detection of high activity has been used to isolate stem-like cells from several different cancer types. However, the expression of the ALDH protein has not been used extensively to study stem cells. It has been reported that ALDH has 19 different isomers expressed in humans that may potentially be responsible for the ALDH activity. This has been the case in the haematopoietic system where it is suggested that ALDH2, ALDH3A1 and ALDH9A1 could account for ALDH activity [158]. Also, evidence suggests that different isomers are expressed in a number of solid cancers that including prostate, breast, lung and colon cancer [158]. Hence the Aldefluor assay might provide a measure of the total activity of different isoforms of the ALDH enzyme. The following result (Figure 3.1) delineates the activity/expression profile of ALDH in three different human colorectal cancer samples.

The average proportion of cells with high activity and expression of ALDH in three colorectal primary tumours was 4 ± 2 % and 4.4 ± 1.8 %, respectively (figure 3.1). Overall, there was no significant difference between the proportion of cells with high activity and high expression of ALDH. Following *in vitro* propagation as spheroids, the activity and expression of ALDH remained similar in primary tissues for up to 4-5 passages (data not shown). It was desired to address whether the cells expressing ALDH1 are the same cells with ALDH$^{\text{high}}$ activity. However, this was not possible to achieve since cells stained with the ALDH antibody had to be permeabilised and fixed in methanol meaning they were no longer viable and so could not then be used in an Aldefluor assay as this requires living cells. Although the antibody used for assessing ALDH expression was raised against the ALDH1 immunogen, there is always a possibility of cross reaction between different isoforms of the ALDH enzyme. Therefore, the activity and expression profiles of ALDH depicted here could be indicative of the activity and expression of the different isoforms of the enzyme taken together. Nevertheless, as the activity and expression of ALDH was similar, both characteristics of ALDH could be used for isolating or as efficacy markers of stem-like cells in primary colorectal tumour tissues.
Figure 3.1. Activity and expression of ALDH1 in primary colorectal cancer. There was no significant difference in the activity and expression of ALDH1 in primary colon tumours. Activity was assessed using the Aldefluor assay, whereas expression was assessed using an antibody that detects intracellular ALDH1 protein. FACS dot plots represent both ALDH\textsuperscript{high} (P5 – gate) and ALDH\textsuperscript{low} (P4 – gate) cells. Values represent the mean ± SEM of ALDH activity in primary colon cancer samples from three different patients.

3.3. Verifying epithelial origins of cancer stem-like cells in primary colorectal tumour and normal tissues

To further characterise ALDH\textsuperscript{high} and CD133\textsuperscript{+} cells, both tumour and normal colon cells were stained using an antibody to epithelial cell surface antigen (ESA, CD326) (figure 3.2). There was significant inter sample variability in ESA staining for both normal and tumour cells. Colon cancer samples were 30-90 % ESA positive, whereas cells from normal tissues ranged from 7-40 % ESA positive. Normal tissues had a lower epithelial cell population owing to the presence of a more complex tissue architecture comprising different cell types, compared to cancer tissues. However, all ALDH\textsuperscript{high}, CD133\textsuperscript{+} and ALDH\textsuperscript{high}/CD133\textsuperscript{+} cells were found to be subpopulations of the ESA\textsuperscript{+} fraction, for both
normal and tumour tissues. Hence, both normal and cancer stem-like cells represent epithelial progeny. This observation is consistent with several reported studies that have shown more than 90% of cancer stem-like cells, represented by either CD133+ [78] or CD133/CD44+ [159] are positive for epithelial cell markers.

**Figure 3.2.** Representative FACS dot plots showing ALDH<sup>high</sup> and CD133<sup>+</sup> cells as subpopulations of epithelial cell surface antigen (ESA) positive cells in primary colon tumour cells. ALDH activity and CD133 expression is restricted to ESA<sup>+</sup> cells only. Gates P8 and P9 represent ESA<sup>-</sup> and ESA<sup>+</sup> populations, respectively. The FACS dot plot on the left is exclusively gated on the P8 population whereas the dot plot on the right is gated on the P9 population. Single cells from colon tumour specimens were stained for CD133 and ESA expression whilst ALDH activity was assessed using the Aldefluor assay. Appropriate controls were included with the ALDH inhibitor and unstained cells were used to gate out any background staining (data not shown). The gates for all dot plots were fixed for a particular cell type analysis to avoid any gating bias. For detailed information on staining conditions see Method Sections 2.5 and 2.6.
3.4. Staining of fresh and frozen primary colorectal cancer cells with cancer stem-like cell markers

It was thought that it would not always be possible to analyse and process patient samples immediately after collection as tissues were often collected in the late afternoon/evening. Therefore, it was important to assess the effects of freezing samples to determine whether this altered the profiling results obtained by FACS analysis of stem-like markers. Single colorectal cells from freshly processed normal and tumour tissues were analysed for their ALDH activity and CD133 expression (Table 3.1). An aliquot was also frozen and these were analysed at a later date; this comparison was performed for 3 patients. The fresh samples contained 3.4 ± 1.9 % and 1.7 ± 0.15 % ALDH\textsuperscript{high} cells in normal and cancer tissues, respectively. CD133\textsuperscript{+} cells were not present in normal fresh samples, whereas fresh cancer tissues had 11.7 ± 2.3 % CD133\textsuperscript{+} cells. Again, ALDH\textsuperscript{high}/CD133\textsuperscript{+} cells were absent in fresh normal tissue. Fresh cancer tissues had an ALDH\textsuperscript{high}/CD133\textsuperscript{+} population of 0.5 ± 0.15%. The normal frozen cells contained a population of 2.2 ± 0.5 % ALDH\textsuperscript{high} cells with 0.06 ± 0.1 % CD133\textsuperscript{+} and no ALDH\textsuperscript{high}/CD133\textsuperscript{+} cells. Analysis of frozen cells isolated from cancer tissue revealed a non-significant increase in the CD133\textsuperscript{+} population to 17.4 ± 8.0 % compared to fresh samples, while the ALDH\textsuperscript{high} (1.5 ± 0.3 %) and ALDH\textsuperscript{high}/CD133\textsuperscript{+} (0.4 ± 0.6 %) double positive populations were similar in size to those detected in freshly analysed tumour cells (Table 3.1). However, with frozen samples there was a greater inter sample variability in ALDH activity and CD133 expression. This could have been due to a different degree of cell viability between samples as the duration of storage was different for each sample, and ranged from 2-8 weeks. Overall, there was a considerable degree of congruence between the stem-like profiles of frozen and fresh samples, reflecting that freezing cells does not alter cellular parameters to an extent that causes significant changes in ALDH activity or CD133 expression. However, since cell viability may decrease with prolonged periods of storage, it was decided that primary cells should be analysed fresh whenever practically achievable or else, as soon as possible after freezing, up to a maximum period of 2 weeks.
Table 3.1 Comparative staining profile of fresh and frozen primary colon tissues

<table>
<thead>
<tr>
<th>Staining parameters</th>
<th>Normal (n=3)</th>
<th>Cancer (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh samples</td>
<td>Frozen samples</td>
</tr>
<tr>
<td>ALDH&lt;sup&gt;high&lt;/sup&gt; activity</td>
<td>3.4±1.9</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.06±0.1</td>
</tr>
<tr>
<td>ALDH&lt;sup&gt;high&lt;/sup&gt;/CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Notes: ALDH activity was measured using the Aldefluor assay. CD133 expression was assessed by antibody staining. Cells were frozen for 2-8 weeks before being analysed. Each experiment was done once for a total of three patient samples. Normal and cancer tissues were obtained from each patient. All % population indicated are a subpopulation of ESA<sup>+</sup> cells.

3.5. Profiling of stem-like cell markers in primary colorectal tumours

To determine whether there were gender-related differences in the profile of stem-like cells in colorectal cancer tissue, patient-derived samples were analysed for ALDH activity and expression of CD133 within the epithelial (ESA<sup>+</sup>) population (Figure 3.3).

The average proportion of CD133<sup>+</sup>/ALDH<sup>low</sup>, ALDH<sup>high</sup>/CD133<sup>−</sup> and double (ALDH<sup>high</sup>/CD133<sup>+</sup>) positive cells in male subjects was 6.96 ± 2.36, 4.57 ± 0.98, and 0.29 ± 0.12, respectively. A similar pattern of markers was observed in female patients. Cells expressing CD133 constituted the largest stem-like fraction (5.92 ± 2.18 %), followed by ALDH<sup>high</sup> cells (2.84 ± 0.50 %) and the double positive population (0.47 ± 0.15 %). For both male and female subjects the double positive (ALDH<sup>high</sup>/CD133<sup>+</sup>) population was significantly lower (p< 0.05) than either of the single stained populations. However, there was no significant difference in the marker profile between male and female patients. This suggests that it is possible to combine data from male and female patients in subsequent analyses.
Figure 3.3. Relationship between gender and profile of stem-like cells in primary colorectal cancer tissue obtained from patients. Samples were grouped based on gender and their corresponding ALDH$^\text{high}$ activity and CD133 expression in the ESA$^+$ cells. Values represent the mean ± SEM, of 23 male and 23 female patients. Refer to table 7.1 (appendix) for patient details.

3.6. Marker profile in normal, adenoma and cancer tissues

To investigate the levels and variation in stem-like markers in human colorectal samples, single cells obtained from primary adenomas and cancers, along with matched normal tissue were FACS profiled for ALDH activity and CD133 and ESA expression (Figures 3.4 & 3.5).

The proportion of cells with high ALDH activity was greatest in adenomas, followed by normal colon and primary tumour. In contrast, CD133 expression decreased in the rank order primary tumour, adenoma, and then normal tissue. Double positive populations were mostly observed in adenomas and primary tumours. The ALDH activity is significantly higher in adenomas compared to both normal and cancer samples ($p<0.01$). Also, there is a significant difference in the proportion of CD133$^+$ staining between normal and cancer tissue ($p<0.05$) and between adenoma and cancer ($p<0.01$), suggesting a gradual increase in expression of CD133 with cancer progression. The double positive population is also significantly higher in tumour cells compared to normal cells ($p<0.05$) (Table 3.2, Figure 3.5).
Figure 3.4. Representative FACS dot plots for stem-like markers ALDH activity and CD133 staining in normal, cancer and adenoma cells isolated from primary colon tissues.

ALDH activity and CD133 expression in normal (A), adenoma (B) and cancer (C) cells. Freshly prepared single cell suspensions from all tissues were stained for ALDH activity (1:200) followed by CD133 (1:10). Diethylaminobenzaldehyde (DEAB), an inhibitor of the ALDH enzyme was used as a negative control to gate out any background fluorescence for ALDH activity (D). The unstained cells were used to gate out any background staining for CD133. The number of events recorded was 10,000 for each dot plot. Gates P7, P8, P9 and P10 represent CD133+ only, ALDH^{high}/CD133^+, ALDH^{low}/CD133^- and ALDH^{high} only populations, respectively, in all dot plots. All cells represented in the dot plots are gated off the ESA+ population.
Figure 3.5. Profile of stem-like cells in primary colorectal normal, adenoma and cancer tissues. Single cells from primary colon samples assessed for ALDH activity and CD133 expression. Significant variation is evident in the stem-like marker profile between normal, adenoma and cancer cells. Single cells isolated from patient tissues were analysed for ALDH activity and CD133 expression; only ESA+ cells were included in the analysis. Significant differences between the levels of specific stem-like populations in each tissue type are designated, where * indicates p< 0.05 and ** corresponds to p<0.01. All values represent the mean ± SEM, where n = 32 normal, 40 cancer and 6 adenoma samples. 32 cancer samples had matched normal tissue available for analysis.

Table 3.2. FACS staining profile (% population) of primary colon tissues with ALDH and CD133.

<table>
<thead>
<tr>
<th>Tissue type (% population)</th>
<th>Normal (n=32)</th>
<th>Cancer (n=40)</th>
<th>Adenoma (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESA+ALDH&lt;sup&gt;high&lt;/sup&gt;</td>
<td>4.25±0.73</td>
<td>2.79±0.42</td>
<td>**&lt;sup&gt;SS&lt;/sup&gt;11.81±1.8</td>
</tr>
<tr>
<td>ESA+CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.45±0.17</td>
<td>*7.66±1.83</td>
<td>SS1.18±0.58</td>
</tr>
<tr>
<td>ESA+ALDH&lt;sup&gt;high&lt;/sup&gt;/CD133&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.07±0.03</td>
<td>**0.37±0.09</td>
<td>0.62±0.52</td>
</tr>
</tbody>
</table>

There is a significant difference in CD133 and ALDH/CD133 staining profile between normal and cancer samples. * p< 0.05, **p< 0.01, SS p< 0.01 (between cancer and adenoma).
3.7. Comparison of clinical staging with stem-like marker profile in colorectal cancer samples

The clinical staging of colorectal tumours represents the degree of tumour progression and hence determines the subsequent treatment strategies. Broadly, the clinical staging is based on the TNM (tumour, node, and metastasis) status of the tumour and the higher the staging, the more advanced the tumour is. Hence, clinical staging is reflective of the tumour status with respect to its migration and invasiveness. Therefore, to assess the stem-like cell marker profile at various clinical stages of colorectal tumour development, cells obtained from patients samples at different stages (from I to IV and colorectal liver metastasis) were analysed for ALDH activity and CD133 expression. Normal and adenoma samples were included in the analysis to represent the early stages of disease progression (Figure 3.6).

![Image of Figure 3.6](image)

**Figure 3.6. Changes in the profile of stem-like cells at different clinical stages of colorectal carcinogenesis.** Single cells were isolated from patient tissues and analysed for ALDH activity and CD133 expression. Only cells expressing ESA were included in the analysis. A total of 32 normal tissue samples were analysed, along with 6 adenomas, 2 stage I, 18 stage II, 5 stage III, 2 stage IV samples and 6 colorectal liver metastases. All values represent the mean ± SEM, apart from stages I and IV where only 2 samples were analysed, therefore, just the mean is given.
The proportion of ALDH\textsuperscript{high} cells was largest in the adenoma tissues (11.8 ± 1.8 %) and lowest in samples obtained from patients with colorectal liver metastases (1.2 ± 0.35 %). There was no significant difference between the fraction of ALDH\textsuperscript{high} cells in histologically normal tissue and cancer stages I-IV, where it ranged from 1.5-4.5 %. CD133 expression was highest in the colorectal liver metastasis samples (16.3 ± 6.08 %) and lowest in the adenomas (1.2 ± 0.6 %). Although normal tissues had a higher percentage of CD133\textsuperscript{+} cells than adenomas, there was a general linear increase in CD133 expression from stage I cancer to liver metastasis, with the exception of stage IV tissues, which had a lower level of CD133 expression, compared to stage III. This discrepancy may be attributed to the small sample size as only 2 stage IV samples were available for analysis. Overall, the linear pattern of CD133 expression suggests that it could be a biomarker of disease progression in colorectal carcinogenesis. The double positive (ALDH\textsuperscript{high}/CD133\textsuperscript{+}) population was similar across all stages of disease progression and represented just <1 % of the total ESA\textsuperscript{+} cells.

3.8. Marker profile based on the site of tumour in the colon

The site of tumour incidence in the colon is not restricted to a particular area. The location varies from the right-sided (ascending) colon to left-sided (descending or sigmoidal) colon and rectum. In a study of over 17,000 patients it was shown that patients with right sided colon tumours had a worse prognosis and the tumours were significantly different from the left sided colon tumours clinically and histopathologically [160]. Therefore, the site of tumour incidence in colon cancer could be an important aspect in predicting drug response. Accordingly, to investigate whether there was a variation in the profile of stem-like cells at different sites of the colon, patient samples were grouped as right, left and rectal, based on the location of tumour incidence (Figure 3.7).
Figure 3.7. Stem-like marker profile in colon tumours according to the site of origin. CD133 expression and ALDH activity was measured in single cell suspensions isolated from human tumours. Samples were analysed from 20 and 6 patients with right- and left-sided colon tumours, respectively, and a further 6 patients with rectal tumours. Significant differences between stem-like populations in different tumour sites are designated, where * indicates p< 0.05. All values represent the mean ± SEM.

There was no significant difference in ALDH activity between tumours originating in the right (3.36 ± 0.73 %) or left colon (3.31 ± 0.99 %) and the rectum (1.61 ± 0.56 %). The double positive population (ALDH$^{\text{high}}$/CD133$^+$) was also similar across all tumour sites. However, the proportion of CD133$^+$/ALDH$^{\text{low}}$ cells was much higher in the right sided tumours (9.33 ± 2.74 %) compared to either the left (2.73 ± 1.49 %) or rectal tumours (1.7 ± 0.63 %). The reason for higher CD133 expression could be due to a greater number of higher grade tumours in the cohort of right sided colon samples. This is evidenced by the fact that the right sided colon sample set contained approximately 70 % and 30 % higher numbers of grade II and III tumours, respectively, compared to the samples arising in the left sided colon.

3.9. *In vitro* assessment of the tumourigenic potential of stem-like cells obtained from primary colon tissues

The sphere forming assay has been increasingly used as a model to assess stem-like properties of cells derived from primary cancers [78]. Also, the spheres derived from
primary cancer cells maintain the expression of stem-like markers in culture conditions, as demonstrated by Ricci et al. [78]. Therefore, the ability of stem-like cells isolated from primary colon cancers to form spheres could be a useful in vitro indicator of the self-renewal potential of these cells. Hence, to assess the self-renewal and stem-like properties in our hands, of primary colorectal cells isolated on the basis of ALDH activity and CD133 expression, cells were grown in serum free culture conditions and tested for their sphere forming ability (Figure 3.8)

Assessment of the sphere forming capacity of the different cell populations isolated on the basis of stem-like markers for 12 patient samples revealed that only the double positive (ALDH\textsuperscript{high}/CD133\textsuperscript{+}), and ALDH\textsuperscript{high}/CD133\textsuperscript{-} fractions, along with the bulk cells, produced spheres (Figure 3.8A, B and C shows representative results). The sorted cell populations took longer than bulk cells to form visible spheres, which is probably due, at least in part, to the additional time required for these cells to recover from the stress associated with sorting. Initially, sorted cells were incubated in 5% carbon dioxide, which resulted in no sphere formation. Fresh cells were then incubated in 10% carbon dioxide and this produced spheres after 14 to 20 days. The difference was probably due to the sensitivity of primary cells to pH, since cell lines were able to form spheres when incubated at the lower concentration of carbon dioxide. A more favourable pH in the media at 10% carbon dioxide than at 5% enhanced the growth of primary cells. The cells negative for both stem-like markers (ALDH\textsuperscript{low}/CD133\textsuperscript{-}) did not exhibit sphere growth even after a month (Figure 3.8D) and eventually died for all 12 patient samples tested. The fastest and biggest sphere growth was observed from the ALDH\textsuperscript{high}/CD133\textsuperscript{+} population, while the ALDH\textsuperscript{high}/CD133\textsuperscript{-} cells exhibited a much slower growth than the double positive population. Cells expressing CD133 but with low ALDH activity failed to form spheres, even after 20 days of culture (data not shown).
Figure 3.8. Stem-like cells derived from a primary colon cancer tissue exhibiting growth as spheroids. Images show representative results obtained with a single patient sample. A primary colon cancer sample was made into a single cell suspension, and immediately sorted based on the expression of CD133 and activity of ALDH. The pure populations were plated in ultra-low attachment plates in serum free media supplemented with growth factors (EGF and FGF). Sphere formation was observed only with the ALDH\textsuperscript{high}/CD133\textsuperscript{+} (A), ALDH\textsuperscript{high} (B) and bulk (C) cells. The ALDH/CD133\textsuperscript{−} (D) population did not show any sphere growth, even when cultured for over a month. The ALDH\textsuperscript{high}/CD133\textsuperscript{−} cells were seeded at a much higher density (1-2x10\textsuperscript{5}) than those populations displaying the stem-like markers ALDH\textsuperscript{high}/CD133\textsuperscript{+} or ALDH\textsuperscript{high} (500-1000). The bulk cells (1x10\textsuperscript{6}) were plated directly into low attachment plates without any sorting, for comparison. The growth of spheres was monitored for a period of two weeks then photographs were taken using a NikonTE2000U camera at 20x zoom. The sphere diameters are represented by the longest length along the sphere measured in micrometers. For detailed information on staining conditions, see Method Sections 2.5 and 2.6.

Bulk cells from normal (n > 30) and adenoma tissues (n = 6) also formed spheres (data not shown). However, the growth of spheres was much slower from adenoma and normal compared to cancer cells, with the former taking over a month to produce visible spheroids; this observation correlates with the tumourigenic potential of cancer cells compared to premalignant and normal cells. Within cancer tissues, tumourigenicity as
predicted by sphere formation was highest in the ALDH<sup>high</sup>/CD133<sup>+</sup> population followed by the ALDH<sup>high</sup> cells; the results also suggest that CD133<sup>+</sup> only cells may be unlikely to generate tumours <em>in vivo</em>. However, a proper <em>in vivo</em> assessment of different sub population of cells is essential in reaching any conclusion on the tumourigenicity of these stem-like cells.

### 3.10. Influence of stem-like profile on the sphere forming capacity of primary colorectal and adenoma samples.

To determine whether the size of specific stem-like fractions within a tissue sample influences sphere formation un-sorted bulk cells from 16 cancer patients and 6 with adenomas were plated for sphere growth. A scoring system was devised which took into consideration the time required for sphere growth, where a size ≥ 30µm in diameter was considered to be a sphere. The fastest (1-2 weeks) growing spheres were assigned a score of 4. Subsequently, sphere scores were assigned as 3, 2 and 1 for growth periods of 2-3, 3-4 and > 4 weeks, respectively. Spheres were profiled for ALDH activity and CD133 expression and the proportion of each population was correlated with sphere forming capacity for each patient sample (Figure 3.9).

There was a significant (p< 0.001) positive correlation (r<sup>2</sup> =0.57) between the proportion of ALDH<sup>high</sup> cells and sphere forming capacity for primary cancer samples (Figure 3.9A). However, there was no such correlation with the percentage of CD133 expressing cells (Figure 3.9B). This is consistent with the previous <em>in vitro</em> experiment (refer to Figure 3.8), where ALDH<sup>high</sup>/CD133<sup>+</sup> but not CD133<sup>+</sup>/ALDH<sup>low</sup> cells were shown to form spheres, and suggests that the single positive CD133 population within primary colon cancers does not initiate sphere formation under the conditions employed. There was also no correlation between sphere formation and cells with a ALDH<sup>high</sup>/CD133<sup>+</sup> phenotype (data not shown). Adenoma samples, in comparison to cancer samples, grew spheres at a much slower rate. Also, not many adenoma samples could form spheres. From the regression analysis (Figure 3.9C), there was no clear relationship between ALDH<sup>high</sup> activity and sphere forming capacity for adenoma cells. This may have been due to the fact that in spite of having a high ALDH<sup>high</sup> population compared to the both cancer and normal tissues (Figure 3.6), the sphere forming capacity was limited as these samples constitute pre-malignant cells and hence do not
propagate as readily as a malignant sample, which is further along the carcinogenesis pathway.

**Figure 3.9.** Correlation between sphere forming ability and proportion of stem-like cells in colon cancer and adenoma tissues. Cells were isolated from patient tissues and plated for sphere growth then the following populations were measured and correlated with sphere forming ability: ALDH$^{\text{high}}$/CD133$^-$ (A & C) and CD133$^+/\text{ALDH}^{\text{low}}$ (B) cells from cancer (A & B) and adenoma (C) samples. The scores given were based on the rate at which spheres formed from the samples. A score of 1 = > 4 weeks to grow, 2 = 3-4 weeks to grow, 3 = 2-3 weeks to grow, and 4 = 1-2 weeks or < 1 week to grow. Spheres measuring ≥30µm were considered a sphere. N = 16 and 6 different patients for cancer and adenoma samples, respectively. Cells were plated at a density of 100,000 cells/well of a 6 well plate. Regression analysis was performed to identify associations between the proportion of each cell population and sphere formation; significant correlations are shown by R$^2$ values.
3.11. Tumour passaging in NOD/SCID mice

Maintenance and growth of primary tissues ex vivo is sometimes challenging owing to the sensitivity of the primary cell with respect to its culture conditions in an in vitro environment. Furthermore, in vitro maintenance may sometimes lead to molecular changes in these primary cells and hence the characteristics of the original tumour can be lost through continued propagation. NOD/SCID mice can provide an alternative, in vivo model, through which primary tumours can be propagated on a long-term basis. This in vivo model might provide better growth conditions and allow growth of the tumour without possibly changing the characteristics of the original tissue. Figure 3.10 describes the general steps undertaken for the in vivo propagation of colorectal tumours, beginning with subcutaneous implantation of the original patient tumour into NOD/SCID mice. The aims of the work detailed in this section were to 1) establish patient-derived xenografts for continued propagation of tissues for use in subsequent experiments, 2) assess the efficiency of transplantation using primary tissues and the ability of tissues to be repeatedly passaged, which would provide evidence that the tissue contained stem-like cells, 3) to examine the ability of passaged tissues to recapitulate the structure of the original sample, assessed histologically, and 4) identify how long samples can be passaged for before changes become apparent.

The growth of human tumours in NOD/SCID mice followed a similar pattern for all primary patient samples transplanted. Approximately 40-50% of cancer samples were able to form tumours on the first implantation in mice. Once the tumour grew from the first implant, subsequent passaging in the NOD/SCID mice was 100% successful. Tumour growth was always slowest during the first passage, after which they grew much faster. When they grew, typically, tumours first became palpable at ~4-5 weeks post transplantation for nearly all original patient tissue samples. The morphological features of tumours implanted in the NOD/SCID mice for the patient samples remained similar to the original tumour. This was evident through the H&E staining shown in Figure 3.10C which illustrates similar tumour morphology between the original specimen and the serially implanted tumour at passage 4. These similar morphological features were maintained until passage 8; this was examined over time for 2 primary tumours (data not shown). Table 3.3 shows the primary colon tumour propagation over
several passages in the NOD/SCID mice and also the sphere forming capacity of the tumour cells derived from the original and mouse passaged tumour.

Figure 3.10. Serial passaging of human cancer tissue in NOD/SCID mice. Tumours derived from human colon were implanted subcutaneously into both flanks of the NOD/SCID mice. Subsequently, when tumours reached a size of 17 mm in diameter, they were harvested from the mice and placed in media for mincing (A). Following mincing, a piece of tumour was injected into each mouse and the animals were monitored for tumour growth. (B) Increased growth rate of three patient samples (P048, P050 and P066) with successive serial passaging in NOD/SCID mice. Passage 1 represents the first time human tumours were grown in mice. Tumour volume was recorded weekly over a span of several weeks until they were harvested for further implantation. H&E staining showed similar tumour morphology at passage 4 compared to the original tumour from patient P050 (C). This finding was representative of results obtained with other patient samples.
Table 3.3. Tumour propagation *in vivo* and *ex vivo* sphere forming capacity for patient colorectal cancer samples

<table>
<thead>
<tr>
<th>Tumour/sphere growth</th>
<th>In vivo (tumour growth incidence in NOD/SCID mice)</th>
<th>In vitro (Sphere growth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td>P0</td>
<td>P1</td>
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<td>S1</td>
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S1-S5 indicates different patient samples, P0 is the original tumour and P1-P5 refers to subsequent passaging in NOD/SCID mice. Values given in the table refer to the number of animals in which a tumour was detected out of the total number that were transplanted at each passage. Sphere growth was assessed using the original patient sample (P0) and tumour from a subsequent mouse passage. Sphere growth was scored according to the time required for sphere formation as follows: + >4 weeks, ++ 2-3 weeks, +++ 1-2 weeks, +++++ 1 week or less.

For assessing *in vitro* growth characteristics following serial implantation, cells isolated from tumours recovered from mice were cultured as spheroids. There was a considerable enhancement in the sphere forming capacity of serially implanted tumours for all patient samples, as shown in Table 3.3.

Overall, the results obtained indicate that tumour propagation *in vivo* provides a suitable system for both maintaining a long-term supply of primary cancer cells that retain the original tumour architecture and also improving the efficiency of sphere growth when cells are cultured *in vitro*; both features facilitate the use of patient-derived cells for screening chemotherapeutic or chemopreventive agents *in vitro*, assessing efficacy *in vivo* and conducting mechanistic studies, all of which require a plentiful supply of proliferating cells.
3.12. Effect of serial implantation in NOD/SCID mice on the stem-like marker profile of human colorectal tumours

ALDH activity and expression of CD133 was assessed in the human colorectal tumours following serial implantation to investigate whether the marker profile of the original patient tumour was altered by repeated passaging in NOD/SCID mice (Figure 3.11). This was important to assess the validity of using cells isolated from passaged tumours as a surrogate for the original tumour cells derived from patient tissue. This analysis may also provide information on which stem-like population is responsible for maintaining tumour growth.

FACS profiling of the tumour tissue obtained from serially implanted NOD/SCID mice revealed a similar level of CD133 expression across all the mouse passages (up to passage 3) and the original patient tumour. The average proportion of CD133⁺/ALDH⁻ cells ranged from 11-13 % across different passages but there was a considerable degree of variation between individual patient samples. The average fraction of cells with high ALDH activity (and CD133⁻) varied from 2.5-6.5 % over three passages in the mice with no significant difference between these samples and the original patient tumour. The size of the double positive population (ALDH⁻⁹/CD133⁺) in the original tumour was also maintained over three passages in the NOD/SCID mice. Hence, since serially passaged tumours exhibit similar cancer stem-like cell marker profiles to the original patient tumour, these markers could be used in assessing activity of potential anticancer drugs in targeting cancer stem-like cells \textit{in vivo} in these patient derived tumours. Furthermore, longer term profiling of one patient sample revealed that a consistent pattern of stem-like markers was maintained for up to 6 sequential passages in mice (Figure 3.11B), confirming that \textit{in vivo} propagation of patient colorectal tumours does not affect the composition with respect to stem-like cells.

3.13. Tumour growth in NOD/SCID mice following injection of ALDH⁻⁹ and ALDH⁻¹ cells at different dilutions

Results from the \textit{in vitro} sphere growth assays suggested that ALDH⁻⁹ cells represent a more tumourigenic population than CD133 expressing cells (section 3.9). Additionally, the size of the ALDH⁻⁹ fraction was not altered upon serial passaging, although this
was also the case for CD133 expression. Whilst the double positive population was shown to have high sphere forming capacity, implying it may actually be the most tumourigenic fraction (Section 3.9), it represents such a small percentage of the cells present that it was considered technically challenging to conduct further experiments requiring sorted populations of these cells. Therefore, to assess the in vivo tumourigenic potential of primary colorectal tumours, sorted ALDH\textsuperscript{high}/CD133\textsuperscript{−} and ALDH\textsuperscript{low}/CD133\textsuperscript{−} cells were injected subcutaneously into NOD/SCID mice at dilutions of 4000 and 1000 cells, and animals monitored for tumour growth (Figure 3.12A&B). H&E staining was performed on tumour sections derived from the injection ALDH\textsuperscript{high} cells and compared to the original tumour to ascertain whether they maintained similar morphology (Figure 3.12C, D&E).

The first incidence of tumour growth (when the tumour became palpable) was noted in the 4000 cell ALDH\textsuperscript{high} group, at around 12 weeks post injection (3/3). All 3 mice in the 4000 cell ALDH\textsuperscript{high} group developed tumours by week 17 post cell injections. In animals that received 1000 ALDH\textsuperscript{high} cells, tumour growth was first evident at 16 weeks in one mouse. Another mouse from this group died because of unrelated health problems within a week of the injection and hence could not be monitored for tumour growth, whilst the third mouse didn’t develop a detectable tumour during the course of the experiment (25 weeks). Once the tumour was palpable, the growth rate was similar in both 4000 and 1000 ALDH\textsuperscript{high} cell groups and once detectable it typically took a further 4-5 weeks for the tumour to reach a size where the animal had to be culled. Tumours were not expected to occur in animals injected with ALDH\textsuperscript{low} cells but 2 out of the 3 mice given the higher dose (4000 cells) developed tumours and 1 of the 3 mice injected with 1000 cells exhibited tumour growth. There was however, generally a delay of several weeks before tumours became palpable in these mice compared to those that were injected with ALDH\textsuperscript{high} cells, although the animal numbers were too small to draw definitive conclusions.
Figure 3.11. Profile of cancer stem-like cells in tumours serially passaged in NOD/SCID mice. The level (average ± SEM) of each stem-like sub population (A) in 3-5 patient samples across 3 successive passages in mice. Tumours harvested at each passage were made into single cells and analysed for the presence of stem-like markers, ALDH activity and CD133 expression. P0 indicates the original patient tumour, with P1 being first implantation and so on. N=5 different patient samples at P0 and P1, whilst 4 different patient samples were analysed at P2, and 3 at P3. (B) shows the long-term serial implantation (up to passage 6) of a single patient sample in NOD/SCID mice. All values represent the mean.
Figure 3.12. Tumour growth in NOD/SCID mice implanted with ALDH\textsuperscript{high}/CD133\textsuperscript{+} ESA\textsuperscript{+} cells. Sorted ALDH\textsuperscript{high/low} cells were injected into the right flank of NOD/SCID mice. Either 4000 (A) or 1000 (B) cells of each type were injected per mouse. Once tumours were palpable, growth was monitored for a maximum of 5-6 months. H&E staining showed tumours formed by injection of 1000 (D) and 4000 (E) ALDH\textsuperscript{high} sorted cells have similar morphology to the original patient tumour (C). Tumour diameters were measured once weekly. N=3 for all groups of mice. Each curve represents a mouse. Graphs show only two curves for 4000 ALDH\textsuperscript{low} group as the third mice did not form tumour. For 1000 ALDH\textsuperscript{high/low} group only one mouse formed tumour (2/3 mice in both ALDH\textsuperscript{high/low} group did not form tumour).

The reason for tumour growth in the ALDH\textsuperscript{low} group may be explained by the stringency of gating, which was initially chosen to provide pure populations but also to maximise the number of cells collected for injection (Figure 3.13). Since ALDH activity is detected as a continuum rather than two discrete populations it is likely that with the initial gating strategy adopted, ALDH\textsuperscript{low} sorted cells may have been contaminated with
a number of ALDH\textsuperscript{high} cells sufficient to generate tumours in mice. An alternative explanation is that the ALDH\textsuperscript{low} population may contain a subpopulation of CD133 positive cells which might also be capable of driving tumourigenesis, however, this is perhaps less likely since the CD133\textsuperscript{+} population lacks \textit{in vitro} tumourigenic potential in our experience.

For subsequent experiments, a more stringent gating policy was used to sort cells (Figure 3.13B), which left a bigger distance between the ALDH\textsuperscript{low} (P4-gate) and ALDH\textsuperscript{high} (P5-gate) populations and involved collecting the extremes of the distribution. Using this protocol, tumours developed in 7 out of 8 mice injected with 2000 ALDH\textsuperscript{high} cells but there was no tumour formation in the any of the 6 animals injected with 2000 ALDH\textsuperscript{low} cells (data not shown).

After an initial round of growth in mice the tumours derived from ALDH\textsuperscript{high} sorted cells were serially implanted in new NOD/SCID mice to assess subsequent growth of these tumours \textit{in vivo}, as shown in Figure 3.14.
Figure 3.13. Gating strategies used for sorting ALDH\textsuperscript{high/low} cells. The original gating parameters used for injecting both 4000 and 1000 ALDH\textsuperscript{high/low} cells in the NOD SCID mice, placed the emphasis on maximising cell yield following sorting (A). Subsequent experiments used more stringent gating parameters to ensure that there was minimal chance of contamination of the ALDH\textsuperscript{low} population with ALDH\textsuperscript{high} cells (B). P4 and P5 indicate the ALDH\textsuperscript{low} and ALDH\textsuperscript{high} cells, respectively.
Figure 3.14. Growth of serially implanted tumours originally derived from tumours generated by injection of 1000 or 4000 ALDH$^{\text{high}}$ cells. Passage 1 refers to the 1st injection of tissue taken from tumours that arose in mice following injection of ALDH$^{\text{high}}$ cells. Passages 2 and 3 arose subsequently from injection of an intact piece of tumour tissue from passage 1 and passage 2, respectively.

The time to first palpable tumour was much earlier for the serially implanted tumours (3-4 weeks, Figure 3.14) than the tumours derived from injection of sorted cells (Figure 3.12). This could be attributed to the fact that the latter approach required sorting of cells, which incurred mechanical stress. As a result of sorting, the cells injected might have taken extra time to recover and a fraction may have died, thus leading to delayed tumour occurrence. On the other hand, for serial implantation tumour pieces were...
injected directly into the NOD/SCID mice. As these tumour pieces did not undergo any processing, the cells avoided any additional mechanical stress and formed tumours more readily than the sorted population. Also, injection of tumour tissues ensured the tumour microenvironment was intact as opposed to the single cell injection. The serial implantation followed a similar growth pattern to passaging of the original tumour (Figure 3.10B - P066), with tumours from passage 2 and 3 growing faster than passage 1 tumours.

Taken together, the in vitro and in vivo data presented suggest that ALDH\textsuperscript{high} cells represent the tumourigenic population within the tumour mass. As such, ALDH activity can be used as a marker of stem-like cells in primary colorectal tumours and may also potentially serve as surrogate markers of efficacy in treatment or prevention studies aimed at targeting stem-like cells.

3.14. Marker profile of tumours derived from injection of ALDH\textsuperscript{high} cells and their subsequent serial passage in NOD/SCID mice

As shown in Figure 3.11, the marker profile of stem-like cells within serially implanted pieces of tumour tissue in NOD/SCID mice remained consistent, with no significant variation compared to the original patient tumour. To determine whether a pure single population of sorted ALDH\textsuperscript{high} cells was also capable of generating the same mixed profile of stem-like cells tumour samples produced by injection of ALDH\textsuperscript{high} cells and subsequent passage of tissue in mice, were analysed for ALDH activity and CD133 expression (Figure 3.15).

Consistent with the previous results obtained for serially implanted patient samples, the stem-like cell marker profile did not appear to be markedly different between the P0 samples, generated by injection of 1000 and 4000 ALDH\textsuperscript{high}/CD133\textsuperscript{-} cells, and the subsequent P1 passage. The proportion of cells exhibiting high ALDH activity after injection of 4000 cells was 2\% and 2.4 ± 0.33\% for P0 (n = 2) and P1 (n = 3) tumour samples, respectively. Similarly, the tumour arising from introduction of 1000 sorted cells contained 2.1\% ALDH\textsuperscript{high} cells (n = 1) and further passage of this tissue afforded tumours with an ALDH\textsuperscript{high} content of 1.3 ± 0.36\% (n = 3). Similarly, the tumour
derived directly from 4000 ALDH\textsuperscript{high} cells (P0, n = 2) contained 1.5 % CD133\textsuperscript{+} cells, whilst the subsequent P1 passage generated tumours with 1.77 ± 0.54 % CD133\textsuperscript{+} cells (n = 3). Following injection of 1000 ALDH\textsuperscript{high} cells, CD133\textsuperscript{+} expression was 2% and 2.06 ± 0.6 % at P0 and P1, respectively. The double positive population also followed similar activity/expression patterns at both passages, for both cell groups. Hence, irrespective of the cell number injected, a similar marker profile was maintained between tumours derived from injection of a single pure cell population and their subsequent serial implantation.

Figure 3.15. Profile of cancer stem-like cells in tumours arising from injection of ALDH\textsuperscript{high} cells and subsequent passage of tissue in NOD/SCID mice. Single cells from a human tumour sample were FACS sorted on the basis of ALDH activity and CD133 expression. Isolated ALDH\textsuperscript{high} cells (1000 or 4000) were implanted into the right flank of NOD/SCID mice, then the resulting tumours were harvested, a sample of tissue was implanted into a further 3 mice and another portion was FACS profiled. P0 represents tumours derived from isolated ALDH\textsuperscript{high} single cells implanted in NOD/SCID mice; data presented are from one mouse for 1000 and 2 mice (average) for 4000 ALDH\textsuperscript{high} cells. P1 represents tumours derived from subsequent serial implantation of this tumour tissue into 3 mice. Values for P1 represent the mean ± SEM.
3.15. Marker profile of primary colorectal tumour cells following *in vitro* culture as spheroids

Spheroid culture selectively promotes the growth of stem-like cells. The differentiated cells cannot survive alone or initiate spheroids under the particular growth conditions used. However, once a sphere starts to grow, it consists of a heterogeneous population of cells from different lineages. As such, long-term *in vitro* propagation as spheres could potentially change the marker profile of stem-like cells from the original tumour. To examine this possibility the variation in ALDH activity and CD133 expression was assessed in spheres over 7 passages (Figure 3.16).

The level of ALDH activity and CD133 expression for the sample shown (n = 1) in figure 3.16 was similar to the original tumour until passage 4, after which there was a visible enrichment of cells with high ALDH activity at passage 7, whereas the fraction of CD133\(^+\) cells generally remained similar to the original tumour, even at passage 7. However, another patient tumour sample that grew until passage 8 did not exhibit any enrichment in ALDH activity (data not shown), so this effect was evident in only 1 out
of the 2 samples analysed. The double positive population remained consistent in size for both samples analysed, until passage 7. Taken together, the enrichment of ALDH activity, especially for samples that were not passaged in NOD/SCID mice could be sample specific as there were different results for two different samples, although more samples needs to be analysed to confirm this. Also, there is a possibility that long-term sphere culture could alter culture conditions that favour the generation and survival of cancer stem-like cells rather than differentiated cells. Given the results obtained, it was decided that for future *in vitro* experiments aimed at assessing the activity of chemopreventive agents, cells derived from early sphere passages (<4) should be used, as these appear to best represent the original patient tumour, in terms of stem-like cell composition.
3.16. Discussion

The percentage of ALDH\textsuperscript{high} cells in primary human colon tumour samples appears to be reportedly differently by different laboratories. Chu \textit{et al.} \cite{159} and Deng \textit{et al.} \cite{161} described a level of 5-40\% and 9.5±7.6\% ALDH\textsuperscript{high} cells in colon tumours from 4 and 5 patients respectively, whereas in the present study which involved 40 patients, the value was much lower, at 2.79 ± 0.42\% (Table 3.2). The percentage of cells with high ALDH activity in normal colon tissue as reported by Huang \textit{et al.} (5.4 \%) \cite{89} was close to the finding in this study (4.25±0.73\%). The use of ALDH activity for identifying stem-like cells in premalignant conditions has been reported for the colon, where ALDH\textsuperscript{high} cells isolated from chronic ulcerative colitis patients were able to progress into adenocarcinomas in NOD/SCIDs mice and also exhibited sphere forming capacity \cite{92}. In the present study, there was a high incidence of ALDH activity in adenoma samples compared to those from cancer patients and matched normal tissue. As such, it is possible that the ALDH\textsuperscript{high} fraction in adenomas could be driving the transition to cancer. However, primary adenoma cells could not be successfully propagated in the NOD/SCID xenograft model employed, based on attempts with 3 patient samples (data not shown), even though these cells exhibited sphere formation \textit{in vitro}. Based on the reported low efficiency of tumour development from ulcerative colitis samples (13.6\% - 3/22 patients) in NOD/SCID mice \cite{92}, it is possible that a small proportion of adenoma samples may have the ability to generate tumours \textit{in vivo} but this would probably require the assessment of many more samples to be detected.

The reported percentage of CD133 positive cells in primary colon cancer varies considerably. Ricci-Vitiani \textit{et al.} \cite{78} \cite{77} have stated that 2.5 ± 1.4 \% (n = 19) of tumour cells are CD133 positive, whereas Fang \textit{et al.} \cite{162} demonstrated that more than 20 \% of colon tumour cells displayed CD133 positivity in their cohort of 9 patient samples. In the present study, which included a greater number of patients (n = 40) an intermediate value was obtained, with 7.66 ± 1.83 \% of tumour cells being CD133 positive. This variation in CD133 population could be attributed to certain factors such as the different methods of cell processing, inter laboratory variability, subjective grading of different colon tumours associated with stem cell yield and a natural variation of stem cells in different patient populations. However, with normal colon cells CD133 expression in the primary samples, was similar to that reported by Fang \textit{et al.} (<5 \%) \cite{162}. There was also a trend for increasing CD133 expression with disease
progression across patients with different clinical stages of colon cancer (Figure 3.6). This is in congruence with a published account detailing a significant correlation between CD133 expression and pathological stage in non-small cell lung cancer patients [163].

Use of in vitro spheroid assays revealed that ALDH \textsuperscript{high} cells were capable of forming spheres whereas ALDH \textsuperscript{low} and CD133\textsuperscript{+} cells failed to initiate sphere formation. However, ALDH\textsuperscript{high}/CD133\textsuperscript{+} cells formed spheres most aggressively. These data may lead to the speculation that ALDH\textsuperscript{high} cells are tumourigenic and that the added expression of CD133\textsuperscript{+} in this population could further facilitate sphere growth. ALDH\textsuperscript{high} cells may represent an early progeny in the hierarchy of stem cells, since a proportion of cells derived from spheroids arising from pure ALDH\textsuperscript{high} populations exhibited CD133 expression (data not shown). Furthermore, sphere growth capacity of the primary colon tumour cells significantly correlated with the ALDH activity of these cells, whereas CD133 expression showed no such correlation (Figure 3.9). Although evidence for CD133\textsuperscript{+} cells representing colonic cancer stem-like cells exists, the specificity of this marker has been debated. This has been shown in a study with the HCT-116 colon cancer cell line, where CD133\textsuperscript{-} derived xenografts had a striking enrichment in CD133\textsuperscript{+} cells [164], thus questioning the specificity of CD133 as marker of cancer stem-like cells. In a primary metastatic colorectal model, both CD133\textsuperscript{+} and CD133\textsuperscript{-} cells could initiate tumour formation in xenografts (n = 9 patient samples) [85]. Moreover, metastatic CD133\textsuperscript{-} cells formed more aggressive tumour than CD133\textsuperscript{+} cells and also expressed other phenotypic markers of cancer initiating cells (CD44\textsuperscript{+}, CD24\textsuperscript{-}) [78]. Therefore, the specificity of CD133 as a colonic stem cell marker remains unclear. Hence ALDH activity seems to be a more consistent marker of stemness in primary colon tumours.

Many initial studies aimed at isolating cancer stem-like cells from a variety of malignancies have employed xenograft models to verify the tumourigenicity of specific sub-populations. Serial implantation in xenograft models was also required to confirm that the tumourigenicity of these cancer stem-like cells is preserved over several passages and that tumours at different generations have a morphological resemblance to the original tumour [79]. As such, a similar xenograft model was established in this
project using NOD/SCID mice. Serial implantation was almost 100% successful for patient tumours that could be propagated from the original tumour (Table 3.3). The tumours derived from serial implantation not only preserved the morphological features of the original tumours, but also maintained similar marker profiles for cancer stem-like cells over several passages. However, any cellular, molecular or genetic changes in these tumours over time were not examined in the present study, but this would be worth doing if the passaged tumours were used for more mechanistic studies investigating specific pathways in the future.

The results emanating from the in vivo study involving injection of ALDH\textsuperscript{high} colorectal cancer cells into NOD/SCID mice also suggest that the tumour initiating capacity resides within the ALDH\textsuperscript{high} cells; the lack of tumour formation from the accompanying ALDH\textsuperscript{low} fraction, when isolated using high purity gating, further confirms the specificity of ALDH activity as marker of human cancer stem-like cells. This is in accordance with published studies where injection of as few as 25 ALDH\textsuperscript{high} cells could initiate tumour formation in NOD/SCID mice using primary colorectal cancer tissues, whereas ALDH\textsuperscript{low} cells failed to initiate tumour growth [89]. A consequence of propagating primary colon cancer cells, both as spheroids and in NOD/SCID mice, is that it leads to the enrichment and maintenance of the cancer stem-like cell population. This is evident in a number of studies where there has been an enrichment of cells displaying markers of cancer stem-like cells in vitro [78, 89]. However, this enhancement of the marker profile does not necessarily represent the situation in the original tumour specimen as any enrichment of cells with cancer stem-like properties would alter the profile and may lead to divergence from the original patient sample. As such, when using these markers for assessing the activity of potential anticancer agents targeted against cancer stem-like cells in a primary cell culture model, it is essential that the marker profile reflects that of the original tumour if it has been serially passaged either in vitro or in vivo. This stability was achieved in the primary cell culture model employed in the present study, where the marker profile for cancer stem-like cells, both in vitro (up to passage 4) and in vivo, remained similar to the original tumour (Figure 3.15 & 3.16).
Chapter 4

4.1. Introduction

The use of curcumin as an anti-cancer agent has been well documented. Curcumin itself exerts a plethora of biological activities in a variety of diseases, which indicates a pleiotropic mode of action. As such, curcumin has been suggested, based on preclinical evidence, to be efficacious in a number of cancers, including breast, prostate, gastric, hepatic, ovarian and colon, as well as leukaemia [165]. Curcumin mediates its anti-carcinogenic effects through multiple targets of cellular processes, which affect proliferation, apoptosis, cell cycle arrest, metastasis and angiogenesis [40]. At a molecular level curcumin has been shown to affect several pathways that involve key genes in the adenoma-adenocarcinoma progression, such as APC, TP53, and KRAS [40].

Particularly in colorectal cancer, there have been numerous reports of the curcumin’s activity of curcumin either alone or in combination with chemotherapeutic drugs using both *in vitro* and *in vivo* models. In HT-29 and HCT-15 colon cancer cells, curcumin exhibited antiproliferative effects, associated with cell cycle arrest at the G2/M phase [166]. Curcumin has also been shown to induce apoptosis in colorectal cancer cell lines at 10-20 µM [167, 168]. At the molecular level, curcumin is known to be an inhibitor of lipooxygenase activity and specifically blocks COX-2 activity [169]. *In vivo* studies have also demonstrated the efficacy of curcumin against colorectal carcinogenesis, especially in rodent chemoprevention models. In F344 male rats that were exposed to the chemical azoxymethane by sub-cutaneous injection to induce cancer, chronic administration of curcumin at 0.2 % and 0.6 % in the diet during the promotion/progression phase of carcinogenesis was found to successfully inhibit the incidence and multiplicity of invasive and non-invasive adenocarcinoma of the colon [170]. In another study of curcumin in *ApcMin* mice, a model of colorectal carcinogenesis commonly used to assess chemopreventive efficacy, dietary doses of 0.2 % and 0.5 % significantly reduced adenoma multiplicity by 40 % [31].

Therefore, both *in vitro* and *in vivo* studies present a strong case for curcumin to be effective in preventing, and potentially treating, human colorectal cancer. However, the ability of curcumin to target colorectal cancer or initiating stem-like cells has not been
investigated in detail. Currently, published data supporting a role for curcumin in targeting cancer stem-like cells have mostly come from studies using cells lines. For example, when combined with either 5-FU or oxaliplatin, curcumin decreases the proportion of cancer stem-like cells defined by CD133 expression, in HCT116 and HT-29 colon cancer cells [171]. Another study showed that curcumin in combination with dasatinib decreased the chemo-resistant cancer stem-like cell population in HCT116 and HT-29 colon cancer cells, as evidenced by the reduced number of cells expressing CD133 and having ALDH^high activity [172]. In vivo studies examining the ability of curcumin to target cancer stem-like cells are also lacking at present, both in a therapeutic and a chemopreventive setting.

Hence, the work described in this chapter is concerned with assessing the ability of curcumin to target cancer stem-like cells, both in vitro and in vivo using primary human cells. In vitro experiments conducted include spheroid assays and stem-like cell marker profiling, following curcumin exposure at clinically achievable concentrations. This was performed in both primary patient-derived colorectal cancer and adenoma cells and represents the first instance where effects have been examined in premalignant cells. The in vivo studies were designed to assess the ability of curcumin to target the growth and expansion of stem-like cells in two mouse models (Apc^Min and NOD/SCID) and correlate effects at the cellular level with efficacy. To address the possible mechanistic pathways through which curcumin targets cancer stem-like cells, the expression profile of stem cell related pluripotent proteins was investigated in both human primary cells and a colorectal cancer cell line (Caco2).

4.2. Patient characteristics of samples used for ex vivo sphere studies

All patient samples were obtained surgically from either colorectal tumours or adenomas. For assessing the effects of curcumin on these primary samples, either freshly obtained cells from patients or cells derived from xenografts of the same patient tumour were used. Table 4.1 describes the characteristics of all the patient samples used for ex vivo experiments.
Table 4.1. Patient characteristics of samples used in assessing the activity of curcumin \textit{in vitro}

<table>
<thead>
<tr>
<th>Cancer Samples</th>
<th>Basal ALDH activity (% ESA)</th>
<th>Basal CD133 expression (% ESA)</th>
<th>Cancer stage</th>
<th>Gender</th>
<th>Tumour site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>2.8</td>
<td>0.9</td>
<td>IV</td>
<td>Female</td>
<td>Right</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1.6</td>
<td>2.7</td>
<td>II</td>
<td>Male</td>
<td>Right</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3.1</td>
<td>3.6</td>
<td>III</td>
<td>Male</td>
<td>Left</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adenoma samples</th>
<th>Basal ALDH activity (% ESA)</th>
<th>Basal CD133 expression (% ESA)</th>
<th>Cancer stage</th>
<th>Gender</th>
<th>Tumour site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>17.6</td>
<td>2.6</td>
<td>Adenoma</td>
<td>Male</td>
<td>Right</td>
</tr>
<tr>
<td>Patient 2</td>
<td>8.3</td>
<td>0.3</td>
<td>Adenoma</td>
<td>Female</td>
<td>Right</td>
</tr>
<tr>
<td>Patient 3</td>
<td>16.5</td>
<td>0.3</td>
<td>Adenoma</td>
<td>Male</td>
<td>Right</td>
</tr>
</tbody>
</table>

4.3. \textit{In vitro} assessment of curcumin activity in primary colorectal cancer samples

Although cancer cell lines provide a useful tool for preclinical evaluation of candidate drugs and chemopreventive agents, various differences make them considerably removed from the actual \textit{in vivo} situation, which can cause great difficulty in extrapolating results to the clinic and using them to predict patient response. Numerous studies have suggested that cell lines poorly represent the diversity, heterogeneity and drug-resistance occurring in patients [173]. As such, primary cells may be more representative of the original tumour in patients. In this experiment colon cancer cells derived from patient tumours were exposed continuously to curcumin and monitored for sphere growth for a period of four weeks, to assess the ability of curcumin to target cancer stem-like cells in primary colorectal cancer (Figure 4.1).

In all three primary colorectal patient samples, which represent three different stages of colorectal cancer (Table 4.1), curcumin had a significant effect on the number and size of spheres produced. The basal ALDH activity and CD133 expression in these patient
samples showed no correlation with their sensitivity to curcumin. The degree of activity across all patients was not a consistent linear dose response; rather, two out of the three patients exhibited a U-shaped dose response. Sphere numbers were significantly reduced by 30-80% across all three patients, with the largest inhibition observed being an ~80% reduction at 5 µM in patient 1 that had stage IV disease (Table 4.1). The effective concentrations for decreasing sphere numbers across all samples ranged from 0.01-5 µM. Sphere size was also affected by curcumin, but not to the same extent as number, although there was general agreement between those concentrations that inhibited formation and reduced sphere size. Sphere sizes were significantly reduced by 20-40% across the three patients at a concentration range of 0.01-5 µM (Figure 4.1). In 2 out of 3 patient samples, curcumin was most effective at submicromolar concentrations (patients 2 & 3, Figure 4.1). The results therefore suggest that curcumin could be effective at concentrations much lower than levels achievable in colon tissues (~20 µM) following daily dosing with 2.35 g in colorectal cancer patients [174]. The decrease in sphere number and size implies that curcumin is targeting both the cancer stem-like cells and their differentiated progeny in primary colorectal cancer.

4.4. Effect of *ex-vivo* exposure to curcumin on the profile of cancer stem-like cells in primary colorectal cancer samples

As colon cancer stem-like cells can be characterised by high ALDH activity and positive CD133 expression, the effects of curcumin on the proportion of cells displaying these markers was assessed following an identical treatment regimen as described in Section 4.3 above, using the same three patient samples. Primary colon cancer cells were derived from spheroids following curcumin treatment and assessed for changes in their marker profile using FACS analysis.
Figure 4.1. Effect of curcumin on sphere formation by primary colon cancer samples from three patients. Single cells from primary colorectal tumours were plated at a density of 30,000 cells/well in six well ultra-low attachment plates. Curcumin (0.01-5 µM) or the vehicle only (DMSO) was added at a range of concentrations twice weekly for 4 weeks. Following treatment, spheres were counted and the size measured. Incubations were performed with each patient sample on three separate occasions and the analysis was conducted for a total of three patients. Significant differences relative to the solvent control are shown, where * indicates p< 0.05 and ** signifies p< 0.01. Values represent the mean ± SEM. Images illustrate spheres formed at the end of 4 weeks in incubations containing DMSO only (control) and 5 µM curcumin. All sphere counts were done by the same individual who was blinded prior to counting to avoid any bias.
The percentage of cells with ALDH$^{\text{high}}$ activity was greatly reduced in all three patient samples following curcumin treatment. Patient 1 showed a linear dose-response, patient 2 plateaued at concentrations ≥0.1 µM, whilst for the 3rd patient there is a similar reduction at 0.1 and 1 µM, where just 5% of ALDH$^{\text{high}}$ cells remain, but the proportion increases again slightly at the highest concentration whilst remaining significantly reduced, mirroring the U-shaped curve observed for sphere number for the same patient (Figure 4.1).

The expression of CD133 was largely unaltered following curcumin treatment, except for patient 3 where there was a significant reduction at 0.1 and 5 µM. As both sphere growth (number and size) and ALDH activity are significantly inhibited by repeated exposure to curcumin, this suggests that curcumin can effectively target the cancer stem-like cell population in primary colorectal cancers at clinically achievable concentrations.

4.5. *In vitro* assessment of curcumin activity in primary colorectal adenoma samples

Premalignant colorectal tissue provides an ideal model for assessing the chemopreventive efficacy of candidate agents and drugs. Curcumin has been shown to exert anticancer effects in colorectal adenoma cells, however, these studies were predominantly performed *in vivo*, using mouse models [31], and only one account addressed the direct effects on adenomas using a cell line derived from intestinal tumours taken from an *Ape$^{Min}$* mouse *in vitro* [175]. Due to difficulties obtaining and maintaining premalignant cells and the fact that no such lines are commercially available for colorectal adenoma, there are currently no data on the activity of curcumin on human colorectal adenoma cells. Furthermore, nothing is known about the potential of curcumin to target the stem-like cell population in primary adenoma cells. Hence, experiments were conducted; analogous to those performed using cancer cells (Section 4.3), to assess the effects of curcumin on primary human colorectal adenoma stem–like cells. Samples derived from three patients were analysed for sphere growth following curcumin exposure for a period of four weeks (Figure 4.3).
Figure 4.2. Effect of curcumin on the proportion of cells with ALDH$^{\text{high}}$ activity and CD133$^+$ expression in primary colorectal tumour-derived spheres. Single cells from primary colorectal tumours were plated at a density of 30,000 cells/well in six well ultra-low attachment plates. Curcumin (0.01-5 µM) or vehicle only (DMSO) was added at a range of concentrations twice weekly for 4 weeks. Single cells harvested from treated spheroids were analysed for ALDH activity, plus CD133 expression. For each patient sample, triplicate experiments were performed on three separate occasions and tissue from a total of three patients was used. Significant differences relative to the solvent control are shown, where * indicates p< 0.05 and ** signifies p< 0.01. Values represent the mean ± SEM.
Following curcumin exposure both sphere number and to a lesser extent, size, were compromised in the primary adenoma cells for all three patient samples. The basal ALDH activity and CD133 expression didn’t exhibit any correlation with the sensitivity of these cells to curcumin (Table 4.1). The effective concentration for sphere number reduction varied between patients and ranged from 0.01-5 µM. Similar to the samples from colon cancer patients (Section 4.3), there was evidence of a U-shaped dose-response for sphere number in two of the patients (1 and 2) with the optimum concentration, showing activity in all patients, being 0.1 µM. Sphere numbers were reduced significantly by 20-50% across all three patients. The largest effects were apparent in patients 1 and 2 where there was a decrease in sphere number by 50% at 0.01 µM and 0.1 µM. The least effect was observed in patient 3 as only 0.1 µM curcumin significantly reduced sphere numbers. The average sphere size was significantly decreased at almost all concentrations across the three patients, although the degree of size reduction was generally much smaller in magnitude compared to the maximal effects observed on sphere number. In addition, there seemed to be patient differences in sensitivity, with consistent but small (~20%) reductions in subjects 1 and 2 and dose-dependent effects in the third patient, where 5 µM curcumin halved the average sphere size. These results suggest that, similar to the effects observed in primary cancer cultures, based on the reduction in sphere number and size, curcumin can potentially target both the differentiated and stem-like fraction within human adenomas.

4.6. Effect of ex-vivo exposure to curcumin on the profile of stem-like markers in primary colorectal adenoma cells

Primary adenoma cells contain a relatively high proportion of ALDH\textsuperscript{high} cells, which could represent the stem-like compartment in premalignant adenomas. This is substantiated by a study that confirmed that ALDH\textsuperscript{high} cells were responsible for the progression of ulcerative colitis into adenocarcinomas in NOD/SCID xenografts [89]. Also, there was an enrichment of colonic ALDH\textsuperscript{high} cells in the colitis patients compared to normal colon tissues, presenting a strong case for ALDH to be a potential marker of stem-like cells in premalignant tissues. Although adenoma cells could not be
Figure 4.3. Effect of curcumin on sphere formation in primary colon adenoma. Single cells from primary colorectal adenomas were plated at a density of 30,000 cells/well in six well ultra-low attachment plates. Curcumin, or vehicle alone (DMSO) was added at concentrations ranging from 0.01-5 μM twice weekly for 4 weeks. Following treatment, spheres were counted and size measured. For each concentration 3 replicate wells were used. Incubations were performed with each patient sample on three separate occasions and the analysis was conducted for a total of three patients. Significant differences relative to the solvent control are shown, where * indicates p< 0.05 and ** signifies p< 0.01. Values represent the mean ± SEM. Images illustrate typical spheres formed at the end of 4 weeks in incubations containing DMSO only (control) and 5 μM curcumin. All sphere counts were done by the same individual who was blinded prior to counting to avoid any bias.
propagated in NOD/SCID mice, *in vitro* these cells could be maintained as spheroids for a maximum of 3-4 passages. Hence, to assess the effect of curcumin on the stem-like marker profile, the same three primary adenoma samples used in the previous experiment (Figure 4.3) were plated for sphere growth and incubated with curcumin for a period of four weeks. ALDH activity and CD133 expression were then assessed in the treated spheres by FACS analysis (Figure 4.4).

Following curcumin exposure the ALDH activity was greatly compromised in all three adenoma patient samples, although differences were observed in the effective concentrations. Significant reductions were apparent at concentrations as low as 0.01 µM for two of the patients, which actually represented the most active exposure in these cases, reducing the proportion of ALDH\textsuperscript{high}/ESA\textsuperscript{+} cells to less than ~20 %. There was evidence of non-linear dose-response relationships, with 1 µM failing to exert a significant effect in any patient although lower concentrations caused profound reductions and in patients 1 and 3 the highest concentration (5 µM) was again effective, removing over ~90 % of the ALDH\textsuperscript{high}/ESA\textsuperscript{+} cells compared to the control. The dose response for ALDH activity in each patient followed the pattern observed in the sphere growth data. The CD133 expression was also affected in all three patient samples. The maximum effect on CD133 expression was evident in patient 3, where there was dose dependent significant decrease at 0.1 (~65 %), 1 (80 %) and 5 µM (90 %). Patient 1 and 2 had significant reductions at 1 µM only.

Taking the combined data on sphere growth and changes in stem-cell like profiles, curcumin can be considered to have anticancer activity in premalignant cells, which is highly promising for its potential role as a chemopreventive intervention in colorectal carcinogenesis.
Figure 4.4. Effect of curcumin on the proportion of cells with ALDH$^{\text{high}}$ activity and CD133$^+$ expression in primary colorectal adenoma-derived spheres. Single cells from primary colorectal adenomas were plated at a density of 30,000 cells/well in six well ultra-low attachment plates. Curcumin (0.01-5 µM) or vehicle only (DMSO) was added at a range of concentrations twice weekly for 4 weeks. Single cells harvested from treated spheroids were analysed for ALDH activity, plus CD133 and ESA expression. For each patient sample triplicate experiments were performed on three separate occasions and tissue from a total of three patients was used. Significant differences relative to the solvent control are shown, where * indicates p< 0.05 and ** signifies p< 0.01. Values represent the mean ± SEM.

4.7. DCAMKL-1, a putative stem cell marker in $Apc^{\text{Min}}$ mice, to assess efficacy of curcumin in targeting cancer stem-like cells in vivo

The $Apc^{\text{Min}}$ mouse represents a suitable in vivo model of colorectal carcinogenesis for testing the chemopreventive efficacy of various agents under consideration. The adenomas develop mainly in the small intestine of the mice with very few in the colon. However, at the molecular level it represents human colorectal cancer. The gut epithelium is subject to a continuous self-renewal process, which suggests a possibility
for the presence of certain cell populations with the inherent capacity to self-renew. These cells within the small intestine possess the characteristics of adult stem cells, giving rise to transit amplifying cells, which can further differentiate into all mature cell types required for gut function. Examining how these cancer stem-like cells are affected by intervention or treatments in pre-clinical models and humans may provide information about potential mechanisms of efficacy and could highlight biomarkers that could be used as intermediate endpoints. In order to assess the effects of curcumin on cancer stem-like cells in vivo, an immunohistochemistry technique was developed to determine the position and number of stem cells in the colon and small intestine of ApcMin mice at various stages of their development, using a putative stem cell marker, doublecortin and CAM kinase-like-1 (DCAMKL-1) [94].

The expression of DCAMKL-1 was evident in cells within the small intestine of ApcMin mice, with different sections of the intestine exhibiting similar staining patterns. Analysis was also performed on intestines from wild-type (WT) adult mice, and these showed occasional single cell staining in the crypt-villi junction and the crypt. The expression of DCAMKL-1 was predominantly cytoplasmic for both WT and ApcMin mice (Figure 4.5). The pattern of staining in the ApcMin mice was different compared to the wild types. Although there was single cell staining in the crypts similar to the WT, there was a trend towards increased number of cells expressing DCAMKL-1 in the villi. It is not clear whether this is due to expression of DCAMKL-1 in the villi of stem cells or loss of crypt niche restriction in DCAMKL-1 expressing cells in the ApcMin mice. It is also not known whether this increase in the number of DCAMKL-1 expressing cells occurs gradually during the course of adenoma development in the ApcMin mice. Therefore, to verify the distribution and number of DCAMKL-1 positive cells in the intestine of these mice, DCAMKL-1 expression was investigated at different developmental stages of ApcMin mice, as shown in Figure 4.6.
Figure 4.5. Representative staining of small intestinal stem cells with an antibody to doublecortin and CAM kinase-like-1 (DCAMKL-1) in $Apc_{Min}$ and wild-type C57/BL6 mice aged 16 weeks. The left panel shows positive DCAMKL-1 staining in the crypts (A) and villi (C) of an $Apc_{Min}$ mouse. The right panel shows cryptic (B) and villous (D) DCAMKL-1 staining in the small intestine of a wild-type C57/BL6 mouse. Red arrows indicate stem-like cells expressing DCAMKL-1. All photographs were taken at 40X zoom with a Leica imaging system.
Figure 4.6. DCAMKL-1 staining in the small intestine of \textit{Apc}^{Min} mice at different developmental stages. \textit{Apc}^{Min} mice maintained on a standard diet were culled at each time point, the small intestine flushed and made into swiss rolls then fixed in formalin, to perform immunohistochemistry for DCAMKL-1 expression, as outlined in Section 2.10.3. For each section, approximately 30-40 villi and crypts were counted and analysis was performed blind. ## and ** indicate a significant difference between the number of DCAMKL-1 positive cells per crypt and villi in mice aged 16 weeks compared to all other time points, respectively, where \( p < 0.01 \). A significant difference (\( p < 0.05 \)) between the average number of DCAMKL-1 positive cells in the villi of 12 week old mice compared to those at 5 weeks is shown by \(^\dagger\). \( N=3 \) mice for each time point. Values shown are the mean ± SEM.

There was an age dependent increase in the number of DCAMKL-1 positive cells in the villi within the small intestine of the \textit{Apc}^{Min} mice. The maximum incidence of these cells was observed at 16 weeks (1 cell per villi), whereas at 5-12 weeks the frequency of DCAMKL-1\(^+\) cells ranged from 0.1-0.4 per villi. In the crypts, there was no age related increase in the DCAMKL-1\(^+\) cells, until 16 weeks of age where a marked 5-fold increase in the frequency was detected compared to 12 week old animals. The DCAMKL-1\(^+\) cells were localised at the +4 region in the crypts of mice at all ages, but with increasing age, there was an evident expansion and movement of these cells up the crypts and into the villi. The higher incidence of DCAMKL-1\(^+\) expressing cells in the villi of older mice suggests that with gradual adenoma development the stem-like cells, as represented by DCAMKL-1\(^+\) staining, exhibit probable migratory characteristics as they populate the villi by moving out of the crypt niche. Another possibility is that these
cells are distinct sub-population of cells that are present in mature intestinal villi of \(Apc^{Min}\) mice, and may represent a more differentiated progeny.

Previous experiments conducted in our laboratory using \(Apc^{Min}\) mice have demonstrated the chemopreventive efficacy of curcumin following dietary interventions at doses of 0.2% and 0.5%, where adenoma multiplicity was significantly reduced by 40%. At lower doses of 0.1% there was no effect on adenoma multiplicity [31]. This historical data coupled with the demonstrated activity of curcumin in human colon cancer and adenoma samples, as shown in Sections 4.3 and 4.5, prompted investigation of the ability of curcumin to target the stem-like population \(in \, vivo\), using the same treated \(Apc^{Min}\) mice, and DCAMKL-1 expression as a marker. Figure 4.7 illustrates representative immunohistochemical staining and quantitation of DCAMKL-1\(^+\) cells in the small intestine of the \(Apc^{Min}\) mice following dietary administration of curcumin.

There was a significant reduction in the number of DCAMKL-1 positive cells in both intestinal crypts and villi of \(Apc^{Min}\) mice, at all doses of curcumin. In mice that received the highest dose (0.5 %) the number of DCAMKL-1\(^+\) cells per crypt was reduced by over 80 %, whilst at both 0.1 and 0.2 %, there was a similar, but lesser, 33 % reduction. In the villi, the maximum effect was also observed with 0.5 % curcumin, with a greater than 80 % decrease in DCAMKL-1\(^+\) cells. As with the crypts, again both lower doses (0.1 and 0.2 %) caused similar effects on the DCAMKL-1\(^+\) cell population within intestinal villi, with significant reductions of ~50 %. These results are consistent with the \(in \, vitro\) adenoma data (Section 4.5), and indicate that curcumin is able to target the premalignant stem-like cell population \(in \, vivo\). To investigate whether curcumin modifies the stem-like population in normal intestinal tissue \(in \, vivo\) or just targets cells harbouring \(Apc\) mutations, wild-type mice were administered dietary curcumin at a dose of 0.5 % for a 16 week period using an identical protocol to the \(Apc^{Min}\) mouse study. As shown in Figure 4.7B curcumin did not significantly alter the number of DCAMKL-1\(^+\) cells in either the villi or crypts of the small intestine in the wild-type animals, suggesting the effects may be restricted to initiated cells. The \(in \, vivo\) efficacy of curcumin in \(Apc^{Min}\) mice may therefore be exerted, at least partly, by selective targeting of the tumour initiating or cancer stem-like populations.
Figure 4.7. Ability of dietary curcumin to target DCAMKL-1 positive cells in *Apc<sup>Min</sup>* (A) and wild-type (B) mice. *Apc<sup>Min</sup>* and wild-type mice were fed on either curcumin or control diet from weaning until 16 weeks of age, following which they were culled and the small intestines flushed and made into swiss rolls, then fixed in formalin, to perform immunohistochemistry for DCAMKL-1 expression, as outlined in Section 2.10.3. For each section, approximately 30-40 villi and crypts were counted. *p< 0.05 represents significant differences between the number of DCAMKL-1<sup>+</sup> cells in the crypts of the control and 0.1 %, and 0.2 % curcumin treated mice. **p< 0.01 represents significant differences between the number of DCAMKL-1<sup>+</sup> cells in the crypts of the control animals and those that received 0.5% curcumin. ¶p< 0.05 represents significant differences between the number of DCAMKL-1<sup>+</sup> cells in the villi of the control and 0.1%, and 0.2% curcumin mice, ¶¶p<0.01 represent significant differences between the number of DCAMKL-1<sup>+</sup> cells in the villi of the control animals and those on 0.5% curcumin. N = 5 mice in each treatment group. Values represent the mean ± SEM.
4.8. Efficacy of curcumin in targeting human cancer stem-like cells in NOD/SCID mice

The *in vitro* studies described in sections 4.3 and 4.5 demonstrated that curcumin was able to target human stem-like cells in both primary colon cancer and adenoma samples. However, to ascertain whether this activity translates to human cells *in vivo* a NOD/SCID xenograft mouse model was used. The well-established poor systemic bioavailability of curcumin after oral administration does not seem to greatly impede its activity in the gastrointestinal tract, since relatively high concentrations of the parent compound can be achieved in these tissues, as shown by its efficacy in *Apc*<sup>Min</sup> mice; however, subcutaneous xenografts rely on curcumin delivery to tumours via the blood supply, meaning it is likely that doses higher than 0.5% may be required for activity and these may cause unwanted toxicity [31]. Therefore, rather than simply increasing the dose a phospholipid formulation of curcumin (Meriva), which has been shown to have a 5-fold higher bioavailability than curcumin in rats [176], was used for this study. To assess the ability of curcumin to target human cancer stem-like cells *in vivo*, sorted ALDH<sup>high</sup> cells from a primary colon tumour were injected into NOD/SCID mice that were maintained on a diet containing either Meriva or the phospholipid carrier alone (Epikuron control). The dose selected was based on a previous efficacy study in nude mice where curcumin in the form of Meriva (1.13 %, equivalent to 0.2 % curcumin) given in the diet [177] was able to significantly lower tumour burden in mice injected with 1x10<sup>6</sup> HCT116 colorectal cancer cells.

There was a significant increase in the survival of mice on a diet containing Meriva compared to those on control epikuron diet (Figure 4.8A), with the last mouse in the treated group surviving for ~7 weeks longer than the last control mouse. Consistent with this delayed tumour growth Meriva was also efficient in significantly increasing the average time to first tumour palpability by approximately 4 weeks (Figure 4.8B). Comparison of the rate of tumour growth for individual animals also revealed general slower tumour development in mice that had received Meriva, although there were two mice in the Meriva group that grew tumours at similar rates to the control animals. Meriva had no effect on the weight of the mice over the course of the study, which suggests it was not causing any toxicity that stops the mice from eating or causes them to lose weight (Figure 4.8D). Taken together, the results indicate that curcumin, in the
form of Meriva, successfully delayed the occurrence and inhibited the development of tumours derived from primary human cancer stem-like cells, as defined by ALDH<sup>high</sup> activity.

Figure 4.8. Effect of dietary Meriva on tumour development in NOD/SCID mice following injection of 2000 ALDH<sup>high</sup> cells. Survival analysis by Kaplan-Meir survival curve (A) between Meriva treated and control epikuron mice; t=0 corresponds to when the cells were injected. Time to first tumour palpability in the Meriva treated group compared to control epikuron group; this was analysed blind by an independent assessor (B). The rate of tumour growth for individual control and Meriva treated mice (C). Average (± SEM) animal body weight over time in Meriva treated and control epikuron groups (D). NOD/SCID mice were injected with 2000 ALDH<sup>high</sup> cells (from a primary colorectal tumour specimen), and put on either 1.13% Meriva or 0.9 % control epikuron diet prior to one week of cell injection. Tumour diameter was measured weekly once the tumours started growing. After tumours reached a maximum size (10 % of animal body weight or 17 mm diameter) or became ulcerated, mice were culled and tumours harvested. N = 5 in each group.
In order to further assess whether efficacy in vivo is mediated via effects of curcumin on the stem-like population within human tumours, the marker profile was characterised in the tumours harvested from the NOD/SCID mice. ALDH activity and CD133 expression was compared in single cells isolated from tumours of mice maintained on Meriva-containing diet and animals on control diet, as shown in Figure 4.9.

**Figure 4.9. Effect of dietary curcumin (Meriva) on the stem-like cell marker profile in human tumours derived from the injection of 2000 ALDH\text{\textsuperscript{high}} primary colorectal cancer cells in NOD/SCID mice.** NOD/SCID mice were injected with 2000 ALDH\text{\textsuperscript{high}} cells (from a primary colorectal tumour specimen), and put on either 1.13% Meriva or 0.9 % control epikuron diet prior to one week of cell injection. Tumour diameter was measured once weekly after it became detectable. When the tumours reached a maximum size (10 % of animal body weight or 17 mm diameter) or became ulcerated due to size, mice were culled, tumours harvested and single cell suspensions generated for FACS analysis of ALDH activity plus expression of CD133 and ESA. One mouse in the epikuron group showed ulceration at a tumour diameter of 15 mm, when the animal was culled. Significant differences between the proportion of cells in the treated and control groups are shown, where * indicates p< 0.05, **p< 0.01, ***p< 0.001. Values are mean ± SEM of five animals per group.

The proportion of cells with either high ALDH activity or CD133 expression was significantly reduced, by approximately 55-60 % in tumours isolated from mice in the Meriva group relative to control animals that received Epikuron only. The double
positive population (ALDH$^{\text{high}}$/CD133$^+$) was also greatly compromised in the Meriva group. Hence curcumin seems to be able to efficiently target primary human cancer stem-like cells \textit{in vivo}.

4.9. Assessment of stem cell protein expression following curcumin treatment in primary human colorectal cancer samples

As described in previous Sections (4.3, 4.5 and 4.8), curcumin has demonstrated efficacy in targeting stem-like cells in primary colon cancer and adenoma cells, both \textit{in vitro} and \textit{in vivo}. Curcumin also successfully targeted cells with an ALDH$^{\text{high}}$ and CD133$^+$ phenotype (Sections 4.4, 4.6 and 4.8). Although these effects are encouraging, the actual functional relevance of these markers as they relate to stem cell function and maintenance is not clearly understood. As such, pluripotent stem cell proteins that are involved in self-renewal and differentiation of stem cells provide a functionally relevant target to assess efficacy of potential anti-cancer drugs. Hence a human proteome profiler stem cell array was used to examine the effect of curcumin on several stem cell proteins. However, only results for the most commonly cited stem cell proteins, namely Nanog, Oct4 and Sox2 are reported here; for the complete data set detailing effects on the entire array of stem cell proteins see Appendix, Figures 7.1-7.3. The effect of curcumin on the expression of Nanog, Oct4 and Sox2 was assessed in the following study in three primary colon cancer patient samples that were also used in the previous experiments investigating effects on sphere growth (Figure 4.10). The concentrations of curcumin used were based on the effective \textit{in vitro} concentrations identified in the spheroid assays (Sections 4.3 & 4.5).

Nanog, Oct4 and Sox2 are three main regulators of stem cell pluripotency and they form a complex regulatory transcriptional network to mediate the pluripotency of stem cells (see Chapter 1). Therefore, these proteins were of interest to elucidate any potential mechanisms through which curcumin might target the cancer stem-like cells in colorectal cancer. The effect of curcumin on the expression of these three stem cell proteins was highly variable among the three different patient samples.
Figure 4.10. Effect of curcumin on the expression of stem cell proteins in primary colorectal cancer sphere-derived cells. Single cells from three primary colorectal tumours were plated at 100,000 cells/well in six well ultra-low attachment plates to allow sphere growth. Curcumin was added at respective concentrations twice weekly for 4 weeks. Whole spheres were then harvested, made into single cells and lysed. The expression of Nanog (A), Oct4 (B) and Sox2 (C) were assessed using a human pluripotent stem cell protein profiler kit (for details see Methods Section 2.12). It was only possible to perform a single replicate experiment per patient sample.
Due to inter-patient variability it wasn’t considered appropriate to combine the data for the three individuals. For patient 1, the maximum reduction (60%) was observed for Oct4 at 0.1 µM. Nanog and Sox2 expression were also reduced by curcumin at concentrations of 0.1 and 1 µM for this patient but the effects were relatively small. Cells derived from Patient 2 showed little difference in the expression of proteins at 0.1 µM curcumin compared to control, but at the higher concentration (1 µM) there was a 40% and 20% reduction in Sox2 and Nanog, respectively. Oct4 and Sox2 expression was slightly decreased at 0.1 and 1 µM curcumin in Patient 3, whereas Nanog expression was reduced only at 1µM and the effect was extremely small. In summary, the expression pattern of these proteins was not consistent following curcumin exposure across patient samples, although there was evidence of effects that warrant further investigation. Also, curcumin was found to affect several other stem cell proteins (see Appendix, Figure 7.1), which could potentially be important and warrant further investigations. Any reduction in expression of Nanog, Oct4 and Sox2 could potentially lead to a mechanism of action through which curcumin targets cancer stem-like cells in colorectal tissue. The inconsistent results may be attributed to the fact that curcumin affects the stem-like cells specifically, and any expression changes in this small population are masked by the much higher proportion of differentiated cells in the spheres, which may be unaffected. Also due to limited amounts of sample it wasn’t possible to conduct the experiment or analysis in triplicate and perform statistical analysis. Therefore, the results from this experiment served solely to orientate further mechanistic studies in cell lines, where populations could be sorted into cancer stem-like cells and non-cancer stem-like cells and suitable replicates performed.

4.10. Effect of curcumin on sphere formation in Caco2 cells, a colorectal cancer cell line

Although primary cells provide a better model for efficacy studies, cell lines are often necessary to elucidate mechanistic information for active compounds. This is mainly due to the fact that the stem-like cells represent only a small fraction of the total cell population and as such, isolation of these cells requires lengthy sorting procedures to obtain sufficient quantities for many types of analyses, leading to low viability and difficulties propagating the sorted cells. On the other hand, cell lines can be expanded
quickly and grow more readily than primary cells after sorting, thus providing an alternate platform for conducting mechanistic studies, especially in the stem-like cell population. However, it is necessary to confirm that the functional endpoints identified in the primary cells treated with curcumin translate to the cell line model. Therefore, sphere formation and growth were assessed in Caco2 cells following curcumin exposure, to ascertain whether this cell line displayed similar sensitivity to that observed with primary samples.

**Figure 4.11. Effect of curcumin on sphere formation in Caco2 cells.** Caco2 cells were plated for sphere growth in six well ultra-low attachment plates. Curcumin was added twice weekly at 0.1 and 1 µM for 4 weeks. Spheres were counted (A) and size measured (B) in three separate experiments. Significant differences relative to the solvent control are shown, where * indicates p< 0.05, and ** signifies p< 0.01. All values represent the mean + SEM.
Curcumin was effective in reducing both the sphere number and size (Figure 4.11). The sphere number was significantly decreased at 5 and 0.1 µM curcumin, whereas the reduction with the intermediate concentration of 1 µM failed to reach significance. Maximum reduction (80 %) in sphere number was caused by the highest concentration of 5 µM, whilst exposure to 0.1 µM resulted in a ~30 % reduction. Sphere size was also significantly impaired at 1 and 5 µM curcumin but only by ~10-30 %. As observed with the primary samples, the extent of sphere size reduction was much smaller compared to changes in sphere number. The fact that curcumin could decrease both sphere number and size suggests that both stem-like cells and their differentiated progeny are targeted by curcumin, which was also the case with primary cancer and adenoma samples (Sections 4.3 and 4.5). Also of note is the fact that curcumin, as in the primary samples, exhibited activity even at the low concentration of 0.1 µM. Given the similarity in responses to treatment, Caco2 cells can be considered to represent a suitable surrogate for primary human cells that can be used to elucidate mechanisms of curcumin action in targeting cancer stem-like cells.

4.11. Effect of curcumin on the proliferation of ALDH\textsuperscript{high/low} Caco2 cells

The most commonly used marker of proliferation in tumour tissue is Ki-67. The Ki-67 protein is strictly associated with cell proliferation and is present at all phases of the cell cycle, except during G₀. Ki-67 is present in all proliferating cells, making it a suitable marker to identify the growing fraction of a given cell population [178]. Curcumin in certain malignancies has been shown to be anti-proliferative [40], however, its effect on the proliferation of stem-like cells specifically in colon cancer has not previously been reported. Therefore, to assess whether curcumin could selectively inhibit the proliferation of cancer stem-like cells, Caco2 cells were sorted based on ALDH\textsuperscript{high/low} activity following curcumin exposure and subjected to a FACS staining method using a monoclonal antibody to Ki-67 antigen (Figure 4.12).
Figure 4.12. Effect of curcumin on the proliferation of ALDH^{high} and ALDH^{low} Caco2 cells. Caco2 cells were grown as adherent monolayers with a seeding density of 0.4x10^6 cells/175 cm^2 flask. Curcumin was added to give a final concentration of either 0.1 or 1 μM every alternate day for a week. Following curcumin treatment, cells were harvested and assayed for ALDH activity. Cells, sorted on the basis of ALDH activity were stained for intracellular Ki-67 expression and FACS analysed (for details see Method Section 2.11). The FACS dot plots are representative of all biological replicates. Data represent the mean ± SEM of three biological replicates performed on separate occasions.

The Ki-67 staining index showed no significant difference in curcumin treated Caco2 cells, compared to untreated controls for both the ALDH^{high} and ALDH^{low} fractions.
(Figure 4.12). Hence, the proliferative capacity of cancer stem-like and non-stem-like cells was not detectably altered using this particular marker following curcumin treatment. The results are consistent with the relatively small reduction in sphere size at these concentrations. The effects seen with sphere size (section 4.3 and 4.5) are cumulative, whereas the Ki-67 analysis shows a snap-shot of the situation at a single time point. Therefore, any subtle differences between treatments may be quite difficult to detect reliably, particularly as nearly all the cells are cycling. The proportion of Ki-67 expressing cells was slightly higher in ALDH\textsuperscript{high} compared to the ALDH\textsuperscript{low} cells at all concentrations of curcumin. At 0 µM the fraction of Ki-67\textsuperscript{+} cells was higher in the ALDH\textsuperscript{high} population compared to the ALDH\textsuperscript{low} population by 7.8 ± 2.6 %. Similarly, for 0.1 and 1 µM curcumin the difference was 5.4 ± 0.21 % and 8.9 ± 7.8 %, respectively. However these differences did not achieve statistical significance.

4.12. Expression profile of Oct4, Nanog and Sox2 in Caco2 cells with high and low ALDH activity following curcumin exposure

The proteome profiler data suggested that curcumin may be able to target the pluripotent stem cell proteins (Section 4.9) in primary colon cancer cells. However, the evidence for a significant effect on Nanog, Oct4 and Sox2 was lacking in the proteome profiler data due to the experimental design and large inter-individual variability between patient samples. Therefore, in order to address this question, a more detailed study was required to quantitatively assess the expression of homeobox proteins (Oct4, Nanog and Sox2) within the cancer stem-like population specifically, following curcumin treatment. Hence Caco2 cells were sorted after curcumin exposure, based on ALDH activity and the expression of Oct4, Nanog and Sox2 was determined by Western blotting (Figure 4.13).
Figure 4.13. Effect of curcumin on the expression of stem cell proteins in ALDH\textsuperscript{high} and ALDH\textsuperscript{low} Caco2 cells. Caco2 cells were grown as adherent monolayers with a seeding density of 0.4x10\textsuperscript{6} cells/175 cm\textsuperscript{2} flask. Curcumin (0.1 or 1 µM) was added every alternate day for a week. Following curcumin treatment, cells were harvested and stained for ALDH activity. Sorted ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells were lysed and the expression of Nanog, Oct4, Sox2 and the housekeeping protein actin were assessed by Western blotting (for details see Methods Section 2.13). Three separate biological replicates were performed on different occasions. Data are expressed as % of the ALDH\textsuperscript{high} control and represent the mean ± SEM. Significant differences between the control and corresponding curcumin treated ALDH\textsuperscript{high/low} cells are indicated, where * signifies p< 0.05, and ** represents p< 0.01. Basal levels of Oct4, Nanog and Sox2 were also compared between curcumin exposed and control, untreated cells and significant differences are designated, where § indicates p< 0.05, and §§ signifies p< 0.05. The basal levels of Oct4 and Nanog in ALDH\textsuperscript{high} cells were significantly higher than in the ALDH\textsuperscript{low} cells. Oct4 expression was 2.5-fold lower in ALDH\textsuperscript{low} compared to ALDH\textsuperscript{high} cells, whereas Nanog expression was approximately 4-fold lower in ALDH\textsuperscript{low} cells. Sox2 expression was similar in both cell populations. Curcumin treatment caused a significant reduction in both Oct4 and Nanog expression in the ALDH\textsuperscript{high} cells without causing any significant effect in ALDH\textsuperscript{low} cells. The most pronounced effect of curcumin exposure was on Nanog expression, as evidenced by a ~55 % reduction at...
both 0.1 and 1µM in the ALDH$^{\text{high}}$ population. Oct4 expression was mostly affected at 0.1 µM curcumin where a 40 % reduction was observed in ALDH$^{\text{high}}$ cells, whereas at 1 µM there was a reduction of only 20 %. Sox2 expression was not affected by curcumin at either concentration. The overall reduction in Oct4 and Nanog expression in ALDH$^{\text{high}}$ cells specifically suggests that curcumin may be significantly affecting the self-renewal capacity of the cancer-stem like population of Caco2 cells.
4.13. Discussion

The use of established cell lines in the pre-clinical assessment of potential anti-cancer drugs has been extensive across virtually all types of malignancy. However, in many instances there is a poor correlation between the efficacy of candidate anti-cancer drugs observed in primary patient cells or clinically and activity demonstrated in cell lines [179]. For instance, the response in primary melanoma cells treated with pentamide showed a lower activity of the compound than observed in cell lines [180] which could be a more accurate estimation of the clinical efficacy of pentamide in melanomas as primary cells may be more representative of the original patient tumour. However this needs to be confirmed in an actual clinical trial examining the efficacy of pentamide.

One of the major differences when using cell lines is their high rate of proliferation compared to cancer cells in vivo. Tumour cells in vivo exhibit a much lower proliferation rate than in vitro cell lines, which are often selected for rapid growth with doubling times much shorter than the cancer cells in vivo [181]. Also, cell lines lack the 3D micro environment that plays a major role in tumour growth in vivo. All these conditions undoubtedly affect cellular characteristics and can select for a subpopulation of cells that differ from the predominant cells of a primary cancer, particularly with long-term passaging [181]. Hence, a potentially effective anti-cancer drug, as evaluated in cell lines, may fail to exert similar effects clinically [179]. Thus the testing strategy for pre-clinical evaluation should ideally incorporate the use of primary patient-derived cells to further augment cell line data, as recently highlighted in a study concerning endometrial cancer, where both cell lines and primary cells were used to show that antioxidants blocks the function of proteasome inhibitors [182]. With recent developments in primary cell/tissue culture models, numerous studies have focussed on using such systems for pre-clinical anti-cancer drug evaluation. For example, Eckerle et al. studied the role of BIRC5 in primary neuroblastoma cells suggesting that it might be a useful therapeutic target [183]. Primary breast and ovarian cancer cells were used to assess the effect of combining inhibitors of the mevalonate pathway in these cancers using an ATP-based chemosensitivity assay [184]. In practice, the use of cell lines may be desirable or even necessary at the early stages of drug development, but the practice of incorporating primary cells for lead optimisation seems to produce good results [179, 185]. This aim was achieved in the current study, which involved the use of both
primary colon cancer and adenoma cells, along with the Caco2 colon cancer cell line to evaluate the ability of curcumin to target cancer stem-like cells.

Curcumin showed significant activity in both primary adenoma and cancer samples, as assessed through spheroid assays and cancer stem-like cell marker profiling. Published studies have also indicated that curcumin could potentially target the stem-like component within cell lines. Of note, there are only two studies to date that have investigated the effect of curcumin in combination with standard chemotherapeutic agents on cancer stem-like cells in colorectal cancer [171], [172] and no accounts of the effects of curcumin when used as a single agent in this tumour type. Only Kakarala et al. [101] have shown that curcumin is effective in targeting stem cells in primary breast tissue. This project represents the first instance where the activity of curcumin has been demonstrated in primary human colorectal tumours and adenomas. The effective in vitro concentrations ranged from 0.01-5 µM; sub-micro molar efficacy of curcumin in targeting cancer stem-like cells has not previously been described. Although the study by Howells et al. [177] showed preliminary evidence that curcumin at 5 µM could target stem-like cells, this was achieved in a colorectal cancer cell line model.

The sphere profiling data revealed a significant reduction in the proportion of ALDH\text{high} cells, in both cancer and adenoma samples after curcumin exposure. As described in Chapter 3, ALDH\text{high} cells represent the tumorigenic subpopulation of cells capable of self-renewal and hence could drive the process of carcinogenesis. In breast cancer, ALDH\text{high} cells have been associated with self-renewal capacity and higher numbers correlate with poor clinical outcome [91]. Thus, curcumin could be targeting the self-renewal pathways of cancer stem-like cells. This was further verified by the curcumin-induced reduction in Nanog and Oct4 protein expression detected in the ALDH\text{high} subpopulation of Caco2 cells, since Nanog is involved in stem cell self-renewal [186] and Oct4 acts as a transcription factor for Nanog expression.

Recent studies have shown that DCAMKL-1 can be used as a marker for quiescent intestinal stem cells in adult mice [94]. These DCAMKL-1 positive cells isolated from mouse tissue also formed spheroids in vitro representing stem-like characteristics. Moreover, the DCAMKL-1 positive cells were expressed in the lower two-thirds of the
epithelial crypts and occasionally in crypt base columnar cells (CBCs), in contrast to another putative intestinal stem cell marker, Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor), which is expressed in rapidly dividing CBCs and occasionally in the epithelial crypt (Figure 4.14) [187]. As in the present study, Gandhi et al. found an enrichment of DCAMKL-1+ cells in ApcMin mice compared to wild-type animals, suggesting that neoplastic conditions harbour an enhanced population of cells with stem-like characteristics. As such, DCAMKL-1 has been used as a marker of cancer stem-like cell to assess the chemopreventive efficacy of celecoxib and its ability to target cancer stem-like cells in ApcMin mice. Gandhi et al. [188] demonstrated that the number of DCAMKL-1+ cells was significantly lower (10-35%) in ApcMin mice on a celecoxib containing diet compared to ApcMin mice on control diet. In another study, DCAMKL-1 was used as an efficacy marker in colon cancer cells and in xenograft tissues. Honoikol, a biphenolic compound used in Chinese traditional medicine in combination with ionising radiation (IR) was able to reduce the number of cells expressing DCAMKL-1 in SW480 colorectal cancer cells. This effect also translated to an in vivo xenograft model where tumour growth was impaired following Honoikol and IR exposure, and also reduced the number of cancer stem-like cells represented by DCAMKL-1+ cells [189]. In the current work, curcumin was shown to efficiently target cancer stem-like cells in treated ApcMin mice, as represented by the DCAMKL-1+ phenotype; in this model, the reduction in DCAMKL-1+ cells correlated with chemopreventive efficacy in terms of decreased tumour burden and number. 

In vivo efficacy data of curcumin also revealed the median survival of NOD/SCID mice ingesting dietary curcumin daily was enhanced by approximately 7 weeks and the time to first palpable tumour was delayed by ~3 weeks. This is an impressive outcome with regards to the efficacy of curcumin that could potentially be achieved in humans. Hence, targeting stem-like cells as shown here, by anti-cancer therapeutic or preventive agents could lead to better clinical endpoints. However, the limitations of this study include the small number of NOD/SCID mice used (5 in each group). To achieve a greater confidence and a more reliable conclusion, the present study needs to be expanded to include more mice per group and also extended to other primary patient samples; this is currently underway in ongoing studies beyond the scope of this thesis.
Figure 4.14. Schematic representation of mouse small intestine showing stem cell location.
The small intestine has approximately 15 layers of cells between the epithelial surface and bottom crypt region. The stem cell position is around the 4th cell from the bottom of the crypt. These stem cells form daughter cells which migrate either down to form Paneth cells or upwards to form differentiated goblet cells and enterocytes of the intestinal epithelium. The position of DCAMKL-1 positive cells is the +4 region, whereas Lgr5+ cells lie in the CBC region. Diagram taken from www.ncbi.nlm.nih.gov/bookshelf.

Overall, both the in vitro activity and in vivo efficacy data present strong evidence that curcumin is able to mediate its anti-cancer effects, at least in part, by targeting cancer stem-like cells. Furthermore, the activity observed in premalignant adenomas suggests that curcumin could also exert its chemopreventive effects by targeting the initiated cancer stem-like cell population. Importantly, all these effects can be achieved at clinically attainable concentrations [174].
5. Chapter 5

5.1. Introduction

Results described in the previous chapter demonstrated the efficacy of curcumin in targeting the cancer stem-like population using primary patient-derived cells and in the NOD/SCID mouse model. However, the mechanism(s) through which curcumin could be exerting its anti-cancer effects on these cells needs to be addressed. To this end, Nanog, Oct4 and Sox2 were identified as a group of interacting stem cell proteins that could be involved in one of the many pathways affected by curcumin. Nanog as previously explained is a homeo-domain protein which plays a significant role in regulating the self-renewal of embryonic stem cells. Also, recent studies have indicated that Nanog expression could be a postoperative marker for liver metastasis of colorectal cancer [190]. Increasing evidence indicates that Nanog could be a key transcription factor for driving the process of carcinogenesis in a number of malignancies (see Chapter 1). Hence, the observed effect of curcumin on Nanog expression in primary cancer stem-like cells could be an important lead for finding a potentially novel mechanism of action of this agent.

The role of Nanog as a transcription factor in embryonic cells has been well defined. Recent studies, including genome wide analysis, suggest that Nanog could bind to multiple promoter regions in human ESCs and thus mediate its self-renewal function via effects on a number of downstream transcriptional targets [191-193]. Post-translational modifications of Nanog including SUMOylation [194] have also been recently documented and Nanog is known to exist as a phosphoprotein [191] [195], with phosphorylation potentially stabilising the protein. Moretto-zita et al. [191] revealed that Nanog is phosphorylated at multiple Ser/Thr-Pro motifs; this phosphorylation is crucial for its interaction with another protein, PIN1 (prolyl isomerase protein), and this leads to Nanog stabilisation by preventing ubiquitinase-mediated degradation. In subsequent experiments, Moretto-zita et al. [191] showed that disrupting the Nanog-PIN1 complex impairs the self-renewal capacity of ECSs and also suppresses teratoma formation in immunodeficient mice injected with mouse ESCs pre-treated with a PIN-1 inhibitor; these observations demonstrate the functional importance of Nanog phosphorylation [191].
Work in this chapter is aimed at determining how curcumin reduces Nanog expression. Initially, the potential for curcumin to cause transcriptional inhibition of Nanog, Oct4 and Sox2 was investigated by qRT-PCR. Secondly, the ability of curcumin to physically interact with Nanog was assessed by a novel protein pull-down assay, developed in-house by Dr Robert Britton, then a spectroscopic binding assay, exploiting the inherent tryptophan fluorescence in recombinant Nanog was used to further examine curcumin binding to the protein. To assess the effects of curcumin on the thermal stability of recombinant Nanog protein the melting point of the protein was determined by circular dichroism (CD) spectroscopy in the presence and absence of curcumin. The effect of curcumin on Nanog phosphorylation was also investigated to ascertain whether curcumin affects Nanog stability by interfering with its phosphorylation.
5.2. Effect of curcumin on mRNA expression of Nanog, Oct4 and Sox2

As described in Chapter 4, protein levels of Nanog and Oct4 were significantly downregulated by curcumin exposure in the ALDH\textsuperscript{high} population of Caco2 colon cancer cells. Therefore, to investigate whether the changes in protein levels were due to changes at the transcriptional level, qRT-PCR analysis of Nanog, Oct4 and Sox2 mRNA expression was carried out in Caco2 cells following curcumin exposure (Figure 5.1).

![Figure 5.1](image)

**Figure 5.1. Effect of curcumin on Nanog, Oct4 and Sox2 gene expression.** Nanog (A), Oct4 (B) and Sox2 (C) mRNA expression in Caco2 cells exposed to curcumin (0.1, 1.0 µM or DMSO solvent control) for three times within a span of one week. Following treatment cells were harvested and analysed for gene expression by qRT-PCR. GAPDH and actin housekeeping genes were used to normalise the data. Values represent the average delta CT (cycle threshold) ± SEM. Experiments were performed in triplicate on three separate occasions.

There were no significant differences in the mRNA expression of any of the genes analysed at either curcumin concentration. Therefore, based on the mRNA expression profile of Nanog, Oct4 and Sox2, curcumin failed to exert any detectable effects at the transcriptional level on these stem cell proteins, over the time frame assessed. However, the qRT-PCR analysis was performed on bulk Caco2 cells; this raises the possibility
that the effect of curcumin on gene expression may have been masked if the changes were specific to a subpopulation of cells, as was the case for Nanog at the protein level. Therefore, to examine the effect of curcumin on the stem-like fraction specifically, Caco2 cells were sorted based on their ALDH$^{\text{high/low}}$ activity following curcumin treatment and analysed for gene expression of Nanog, Oct4 and Sox2 by qRT-PCR (Figure 5.2).

**Figure 5.2.** Effect of curcumin on Nanog, Oct4 and Sox2 gene expression in ALDH$^{\text{high/low}}$ Caco2 cells. Cells were sorted based on ALDH activity following curcumin exposure for a week and Nanog (A), Oct4 (B) and Sox2 (C) mRNA expression was determined in both ALDH$^{\text{high}}$ and ALDH$^{\text{low}}$ cells. Values are plotted as average delta CT. GAPDH and actin were used as housekeeping genes for data normalisation. Values represent the mean ± SEM. Experiments were performed in triplicate on three separate occasions.

Nanog, Oct4 and Sox2 all remained unchanged following exposure to either 0.1 or 1 µM curcumin, in both the ALDH$^{\text{high}}$ and ALDH$^{\text{low}}$ cell populations. Overall, these
results suggest that the biological effects of curcumin on cancer stem-like cells could be mediated through the Nanog pathway at the protein, rather than mRNA level.

5.2.1. Effect of resveratrol on sphere formation in primary colorectal cancer cells

Resveratrol is another polyphenolic, naturally derived compound that has been reported to exhibit chemopreventive properties in a wide variety of malignancies (refer to Chapter 1). Recent reports indicate that resveratrol can target cancer stem-like cells in preclinical models of pancreatic and breast cancer, both in vitro and in vivo [196] [197]. Therefore, to assess the ability of resveratrol to target primary colon cancer stem-like cells, sphere forming capacity was measured in patient-derived colon cancer and adenoma cells following resveratrol exposure (Figure 5.3, Figures 7.2 Appendix).

The sphere forming capacity of cells from three different primary colon cancers and one adenoma sample was unaffected by resveratrol exposure, with no significant treatment-related differences detected in sphere number in any patient or size in a single patient sample (figure 7.2D). This lack of effect contrasts with the results obtained for curcumin (Sections 4.3 & 4.5), which inhibited the growth of all adenoma and cancer samples tested. Hence, to assess whether this lack of resveratrol activity could be associated with an inability to affect the pluripotent stem cell markers, the expression of Nanog in ALDH$^{high/low}$ Caco2 cells was evaluated following resveratrol exposure (Figure 5.4).
Figure 5.3. Effect of resveratrol on sphere number (A) and size (B) in primary colorectal cancer cells. Single cells from a primary colorectal tumour were plated at 30,000 cells/well in six well ultra-low attachment plates. Resveratrol was added twice weekly for 4 weeks and following treatment, spheres were counted and the size measured. Data for only one patient sample is shown here. Results from cultures derived from three additional patients (two cancers and one adenoma) are shown in Appendix Figure 7.2. All values represent the mean ± SEM of three replicates performed once.
Figure 5.4. Effect of resveratrol on Nanog protein expression in ALDH\textsuperscript{high/low} Caco2 cells.

Caco2 cells were grown as adherent monolayer cells with a seeding density of 0.4x10\textsuperscript{6} cells/175 cm\textsuperscript{2} flask. Resveratrol (0.1 or 1 µM) was added every alternate day for a week. Following resveratrol treatment, cells were harvested and sorted based on ALDH activity. Nanog expression was then determined in populations of ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells by Western blotting. Three separate biological replicates were performed and data represent the mean ± SEM.
As expected, the expression of Nanog protein following resveratrol exposure remained unaffected; this was evident in both ALDH$^{\text{high}}$ and ALDH$^{\text{low}}$ cells. Hence resveratrol failed to cause any reduction in the pluripotent stem cell marker, Nanog. As such, this might explain why resveratrol could not reduce sphere number in cultures of primary cells. This finding also supports the idea that curcumin is mediating inhibitory effects on sphere formation through the Nanog pathway. However, in order to reach such conclusions, direct experimental proof that Nanog expression is necessary for the growth and expansion of spheres derived from Caco2 cells and colorectal cancer in general would be required. In fact this has been shown in prostate cancer where knock-down of Nanog impaired sphere formation in prostate cancer cells [147].

5.3. Interaction of curcumin with Nanog and Oct4

The mRNA profiles of Nanog and Oct4 in Caco2 cells revealed no significant changes following curcumin exposure. However, protein expression levels of Nanog and Oct4 were significantly reduced in the ALDH$^{\text{high}}$ cancer stem-like cell population of Caco2 cells after curcumin exposure. This suggests that the effects of curcumin on Nanog and Oct4 expression could be at the protein level and may involve changes in protein stability. To investigate whether curcumin might alter protein levels by a direct interaction, a pull-down experiment was conducted to assess whether curcumin-coupled beads could non-covalently bind to Nanog and Oct4 (Figure 5.5).

The pull-down assay revealed that curcumin binds to and pulls both Nanog and Oct4 from whole cell lysates of Caco2 cells (Figure 5.5A and B). Importantly, the negative control beads showed no binding to either endogenous protein, Nanog or Oct4. The crude lysate, which represents the input sample for each incubation, was run as a positive control for both the proteins. The pull-down experiment with the crude lysate however does not confirm a direct interaction of curcumin with Nanog or Oct4. These proteins could potentially be pulled as part of a complex and only one of these proteins or even a completely different protein may be the direct curcumin binder. However, a subsequent pull-down experiment was performed using recombinant Nanog protein (Figure 5.5D) and this confirmed that curcumin actually interacts directly and binds non-covalently to Nanog. It has been reported that the Oct4 and Nanog bind to each
other at the protein level in ESCs [198], hence it is possible that Oct4 is pulled in the crude lysate because it is bound to Nanog rather than curcumin directly. This could be confirmed by doing a pull down experiment with recombinant Oct4 alone. However, at the time this experiment was conducted there was no commercial source of full length, pure recombinant Oct4 available, apart from tagged versions, which may have interfered with the assay.

In an attempt to assess the specificity of the interaction between curcumin and Nanog, resveratrol was used as a potential negative control compound that doesn’t alter Nanog protein expression in vitro. A pull-assay was performed with resveratrol-coupled beads, synthesized with the same linker chain as the curcumin beads, to investigate whether resveratrol could bind endogenous Nanog from Caco2 lysates (Figure 5.5D). The results indicate that there was no binding of Nanog to resveratrol or the control beads. This is consistent with the hypothesis that changes in Nanog expression could be mediated by direct curcumin binding and since resveratrol doesn’t bind and doesn’t affect protein levels (Figure 5.4) it has no activity against cancer stem-like cells in colorectal cancer. To further verify the binding of curcumin to Nanog using an independent technique, a biophysical assay was performed that exploits changes in the intrinsic tryptophan fluorescence of the recombinant Nanog protein as a result of direct interactions (Figure 5.6).
Figure 5.5. Protein pull-down assays with curcumin and resveratrol-coupled beads.

Detection of Nanog (A) and Oct4 (B) binding to curcumin beads incubated with Caco2 whole cell lysates. Detection of full length recombinant Nanog (0.5 ng and 1 ng) binding to curcumin beads (C). Detection of Nanog binding to resveratrol beads (D) incubated with Caco2 whole cell lysates. Incubations with control beads were performed in parallel and included on each gel for the detection of any non-specific binding for both curcumin and resveratrol beads. The crude input lysate (500 µg of protein) was included as a positive control on each gel. Proteins were detected using Western blotting. For detailed descriptions, see Methods Section 2.15.
Figure 5.6. Binding curve of curcumin and recombinant full length Nanog determined by fluorescence spectroscopy. Tryptophans were excited at 295 nm and the resulting fluorescence intensity recorded at 328 nm in samples containing varying concentrations of curcumin. Recombinant Nanog at 40 nM concentration was used for the assay. Both a one and two binding site model was used to fit the data points. The dissociation constants KD₁ and KD₂ represent two available binding sites for curcumin in the recombinant Nanog protein. The experiment was performed at room temperature.

The fluorescence assay provided further evidence that curcumin could bind to the recombinant Nanog protein. Curve fitting revealed that a two-site binding model fits the data points better than a one-site binding model, indicating a 1:2 stoichiometry if both the protein and curcumin were monomeric. The saturation binding concentration of curcumin is 100 nM, after which there is no enhanced binding. This correlates with the most biologically active concentration of curcumin in human cancer stem-like cells (100 nM) identified in Chapter 4.

To further ascertain binding of curcumin to Nanog and determine whether this binding causes any structural changes, the intrinsic tryptophan fluorescence of recombinant Nanog was used to assess the thermodynamic stability of Nanog. Thermal denaturation of the protein was performed in the presence of 100 nM curcumin or vehicle (see Figure 5.7).
Figure 5.7. Effect of curcumin on the thermal stability of Nanog. The melting curve was generated by measuring the intrinsic fluorescence of tryptophan in Nanog against a temperature gradient. Recombinant Nanog at 40 nM concentration was used for the assay. Tryptophan residues were excited at 295 nm and the fluorescence intensity recorded at 328 nm. Curve fitting revealed a melting point of 62°C for Nanog. The melting curve for Nanog+Curcumin could not be fit as the fluorescence intensity of tryptophan failed to reach a saturation point.

The denaturation curve for Nanog (Figure 5.7) yielded a melting temperature of 62°C, which is at the high end for proteins, implying that Nanog is quite stable compared to other short lived proteins [199]. The effect of curcumin on protein stability is not clear because the fluorescence intensity did not plateau at high temperatures and the fit was therefore ambiguous. This could be due to interference, caused by the fluorescence signal of curcumin itself, with the tryptophan fluorescence at high temperatures. To investigate this further, fluorescence intensity was measured at a low (25°C) and a high (93°C) temperature for both tryptophan and curcumin emission ranges (Figure 5.8).

Tryptophan residues were excited at 295 nm, and their emission was measured from 310 to 430 nm. Curcumin was excited at 430 nm, and its emission measured at 450-550 nm. The fluorescence intensity of the tryptophan residues increased with higher temperatures, as shown by the differences in the two spectra in Figure 5.8, which is caused by a change in the environment of the tryptophan residues from hydrophobic to more hydrophilic surroundings and is consistent with unfolding of the protein. However, the emission spectra for curcumin reveal lower fluorescence intensity at 93°C compared to 25°C, which might indicate that curcumin is degrading at high temperatures. Therefore, as curcumin does not seem to be stable at high temperatures,
the interaction with recombinant Nanog might be affected by the thermal degradation of curcumin and as such a thermal saturation point was not achieved at high temperatures, leading to an ambiguous fit, as shown in Figure 5.7.

![Graph](image_url)

**Figure 5.8. Comparison of tryptophan and Curcumin emission spectra.** Emission spectra for tryptophan were recorded at 310 – 430 nm. Curcumin emission spectra ranged from 450-550 nm. The excitation wavelength for tryptophan and curcumin was 295 nm and 430 nm, respectively.

### 5.4. Thermal stability of recombinant Nanog assessed through circular dichroism spectroscopy

The tryptophan assay failed to reveal the effect of curcumin on the thermal stability of Nanog. Hence, an alternative method was required for assessing the effect of curcumin on the thermal stability of Nanog. Circular dichroism (CD) spectroscopy is a method based on the fact that left and right circularly polarised light is absorbed differentially [200] [201]. There are numerous applications of CD spectroscopy; one common use is to predict the secondary structure of proteins [202]. In addition, the technique can be used to evaluate the effect of various physical factors such as temperature, pH and concentration-dependent effects of chemical compounds on conformational changes of
proteins in solution. Here, the methodology was utilised to determine whether the presence of curcumin affected the thermal stability of recombinant Nanog.

CD spectra were collected from 190-260 nm, with a temperature gradient from 23°C to 94°C at a heating rate of 1 degree per minute. Recombinant Nanog was measured both with and without curcumin. Thermodynamic analysis of wavelength spectra recorded at different temperatures showed that the native protein displayed a two-state unfolding, yielding two melting points (Figure 5.9). The first melting point at 49.5 °C indicates a transition from folded state (state 1) to intermediate state (state 2). The second melting point at a temperature of 79.7 °C indicates transition from intermediate folded state to an unfolded state (state 3). For the native protein it takes time to reach state 2 from state 1, whereas the second transition happens quickly (Figure 5.9A). When curcumin was added at a concentration of 0.1 µM to the recombinant protein there was a change in the melting curve of the protein. The first transition occurs at 47°C followed by the second transition at 67.5°C (Figure 5.9B). Therefore, there is a decrease in both melting points suggesting that curcumin could thermodynamically destabilise the protein, since the unfolding occurs at lower temperatures thus requiring less energy to become fully unfolded.

Hence, results from both the pull-down and fluorescence assays give strong evidence of a direct curcumin-Nanog interaction, which could play a role in mediating the biological effects of curcumin in colorectal cancer stem-like cells.
Figure 5.9 Effect of curcumin on thermal stability of Nanog using CD spectroscopy. Native recombinant Nanog (A) showed two transitions from folded (1), to intermediate (2), to fully unfolded (3) state. Curcumin reduced both transition (melting points) temperatures of Nanog (B). The temperature was ramped from 23°C to 94°C at a rate of 1°C per minute. Wavelength spectra using CD were collected from 260-190 nm at 1 nm intervals. Data were analysed by Global3 software. Curcumin (100 nM) was incubated with 83 ng/mL of recombinant Nanog prior to temperature ramping.

5.5. Effect of curcumin on Phospho-Nanog-pSerine71 in ALDH\textsuperscript{high/low} Caco2 cells

Nanog protein expression in Caco2 cells was significantly reduced by curcumin, as shown in Chapter 4. However, the change in protein expression did not appear to be due to a corresponding decrease at the transcriptional level, as evident from the qRT-PCR data (Figures 5.1 & 5.2). Therefore, an alternative explanation for the reduced levels of Nanog protein could be curcumin-induced changes in post translational modifications that may affect protein stability. As such, recent studies have indicated that phosphorylation at specific sites in the Nanog protein sequence, namely the Ser/Thr-Pro motifs, are involved in maintaining the stability of Nanog in human embryonic cells [191]. Hence, to address whether the extent of Nanog phosphorylation is altered by the presence of curcumin, the ratio of total to phosphorylated Nanog at serine 71 was determined in ALDH\textsuperscript{high/low} populations of Caco2 cells exposed to curcumin (Figure 5.10).
As previously observed with total Nanog expression in this cell line (figure 4.13), the basal level of phospho-Nanog in control, untreated ALDH$^{\text{low}}$ cells was significantly lower than in the ALDH$^{\text{high}}$ fraction, at approximately one quarter of the levels detected. Curcumin exposure significantly decreased the amount of phospho-Nanog in the ALDH$^{\text{high}}$ cell population by approximately 60 and 25 % at concentrations of 0.1 and 1 µM, respectively (figure 5.10). In contrast, neither curcumin treatment affected phospho-Nanog levels in ALDH$^{\text{low}}$ cells. However, the reason for the reduced levels of phospho-Nanog in the stem-like fraction after curcumin exposure could have been due to the accompanying lower levels of total Nanog that were also detected in the cells (Figure 5.10). Therefore, to ascertain whether curcumin actually reduced the proportion of phosphorylated Nanog, the ratio of phospho-Nanog/Nanog was calculated (Figure 5.10). The ratio was only significantly reduced by curcumin at 0.1 µM in the ALDH$^{\text{high}}$ cells; the extent of phosphorylation was unaffected in this population at the higher concentration. These results verify that curcumin significantly reduces both total Nanog and the proportion that is phosphorylated at 0.1 µM, however, at 1 µM only the total protein levels were decreased. This is in accordance with the U-shaped dose response relationships described for curcumin activity in primary colorectal tumour and adenoma cells (Chapter 4). Therefore, the reduction in phospho-Nanog caused by curcumin at 0.1 µM could adversely affect Nanog stability by promoting its degradation, resulting in significantly lower levels of total Nanog in cancer stem-like cells.
Figure 5.10. Effect of curcumin on Phospho-Nanog expression in ALDH\textsuperscript{high/low} Caco2 cells.

Phosho (Serine 71) Nanog levels (A), Nanog (B) expression, and the ratio between Phospho-Nanog and Nanog (C) in ALDH\textsuperscript{high} and ALDH\textsuperscript{low} Caco2 cells following curcumin exposure. Curcumin (0.1 or 1 µM) was added every alternate day for a week. Following curcumin treatment, cells were harvested and stained for correct ALDH activity. Sorted ALDH\textsuperscript{high/low} was lysed in lysis buffer and protein concentration determined by the Bradford assay. Protein expression was assessed by Western blotting (for details see Methods Section). Actin was used as a loading control for normalisation. Three separate biological replicates were performed on different occasions. Data are expressed as % of control (mean ± SEM). p<0.05, **p<0.01 for comparison of control and treated ALDH\textsuperscript{high/low} cells. §p<0.05, §§p<0.01 for comparison of basal levels of Nanog in control untreated cells.
5.6. Effect of curcumin on Nanog stability

As shown previously, curcumin affected Nanog phosphorylation (Figure 5.10), which could lead to enhanced proteasome-mediated degradation of the protein. Therefore, a cyclohexamide assay (CHX) was performed on Caco2 cells to assess whether curcumin mediates its inhibitory effects on Nanog by promoting its degradation.

**Figure 5.11. Cyclohexamide assay for assessing stability of Nanog.** Representative Western blots following cyclohexamide exposure in Caco2 cells (A) and subsequent densitometric analysis (B). Caco2 cells were treated with cyclohexamide (CHX) (100 µg/mL) and harvested at various time points. The relatively short lived cyclin D1 was included as a positive control to demonstrate the expected effects of CHX on protein levels. In parallel, incubations were conducted with CHX in the presence of curcumin; cells were pre-treated with curcumin for 1 h prior to addition of CHX. Actin was used to normalise the data. Values are mean ± SEM of three independent experiments.
Cyclohexamide, an inhibitor of protein synthesis, is commonly used to determine the half-lives of short lived proteins in cultured mammalian cells [203]. Accordingly, analysis of cyclinD1 levels as a positive control revealed this particular protein has a very short half-life of around 45 minutes, which is consistent with published results in mouse embryonic fibroblasts (NIH-3T3 cell line) [204] [205] and confirms the assay is working. In contrast, Nanog, was found to be very stable in Caco2 cells; there was no consistent or convincing reduction in protein levels over time, which meant the half-life could not be determined using this approach. Also, following curcumin exposure at a concentration of 0.1 µM, the stability of Nanog was not significantly altered. Although there was a decrease in the total Nanog in cells exposed to CHX and curcumin, compared to the cells exposed to CHX only, this did not achieve statistical significance. The failure to detect the effect of curcumin on Nanog levels could have been due to the relative stability of Nanog in Caco2 cells, as the CHX assay is not suitable for determining the turnover of long–lived proteins.

The results in Caco2 cells were unexpected since published data indicate that Nanog has a half-life of ~2 h in HEK 293 cells, a kidney embryonic stem cell line [206]. Therefore, to further verify that the CHX assay was working and to reproduce the published observations, the CHX assay was conducted in HEK 293 cells.
Figure 5.12. Cyclohexamide assay for assessing stability of Nanog in HEK 293 cells. Cyclohexamide inhibited protein synthesis by 50% after 8 h. HEK 293 cells were treated with cyclohexamide (100 µg/mL) and harvested at various time points. Actin was used to normalise the data. Values represent the mean ± SEM of % Nanog expression relative to control from 3 different experiments performed on separate occasions. Significant differences relative to the Nanog level at t=0 are indicated, where ** indicates p< 0.01.

As shown in Figure 5.12, there was a general decline in protein levels over time, which becomes significant at 8 h, where a 50% reduction is observed compared to protein level at t=0, indicating that the half-life of Nanog in HEK 293 cells was approximately 8 h. However, this was much longer than the half-life of Nanog in HEK293 cells reported in a couple of studies [206, 207].
5.7. Discussion

Usually the level of mRNA expression provides an indication of the protein abundance in a steady state system. However, on perturbation of the cellular system the steady state is breached and as such any correlation between mRNA and protein expression falls as many other parameters come into play that regulate protein abundance. This has been well explained by Marcotte and Vogel in their review article [192]. They mention that mRNA expression accounts for only 40% of the variation observed in the expression of the respective protein [192]. Expression of mRNA could just act in a switch-like fashion to indicate the presence of the protein i.e., if the mRNA expression is low it is unlikely that the protein is present in the system and vice-versa. The remaining 60% variation in protein expression could be attributed to post–transcriptional regulation, post-translational modification of proteins and protein degradation. Once the contribution of transcriptional regulation of protein expression is factored out the major contributors that account for differences in protein abundance are protein degradation and post-translational modifications [192]. Another review suggests that transcription, translation and degradation may couple with each other frequently and form feedback loops to dictate the overall gene and protein expression [193]. The results obtained for mRNA expression in both sorted and bulk cells (Figures 5.1 & 5.2) suggest that the difference in Nanog and Oct4 expression following curcumin exposure could be regulated by protein degradation or post-translational modifications, as discussed above. The lack of evidence for transcriptional regulation of the protein expression changes in the cell system employed further enhances the possibility that curcumin might be targeting Nanog at the protein level and not at the gene expression level. Therefore, the interaction of Nanog and curcumin could potentially have functional consequences and this was investigated in subsequent experiments.

A protein pull-down assay was used to demonstrate the direct non-covalent interaction between Nanog and curcumin. Similar pull-down approaches been used to assess interactions of specific candidate proteins or identify novel target proteins of potential chemopreventive agents. Particularly for resveratrol, several groups have employed pull-down assays to verify interactions with potential target proteins or as a starting point for elucidating novel mechanisms of action that might contribute to efficacy [208, 209]. The results obtained in the present study indicate that curcumin can bind to both
endogenous and recombinant Nanog protein. This was further verified in biophysical experiments where curcumin was demonstrated to have two binding sites within Nanog. As a comparison, a pull-down assay was conducted with resveratrol-coupled beads. Interestingly, resveratrol did not bind to Nanog which implies that binding of curcumin to Nanog could be functionally important since resveratrol also failed to exhibit any \textit{in vitro} activity in spheroid assays and treatment had no effect on Nanog protein expression in primary cells.

Previous studies have shown that interaction of PIN-1 with Nanog in embryonic stem cells prevented Nanog degradation by facilitating phosphorylation of Nanog at specific sites [191]. Initial attempts were made to verify the interaction of Nanog and PIN-1 in Caco2 cells by co-immunoprecipitation but to date these studies have been unsuccessful. As an alternative, the extent of Nanog phosphorylation was compared in control and curcumin-treated cells to investigate whether differences in the serine 71 phosphorylation status of Nanog might account for the reduced total protein levels previously seen in ALDH$^\text{high}$ stem-like cells, as a consequence of reduced stability. The ratio of phospho-Nanog/total Nanog was only significantly reduced at 0.1 \(\mu\)M, not at 1 \(\mu\)M curcumin. Interestingly, the spectroscopic binding experiments also revealed the saturation concentration to be 0.1 \(\mu\)M curcumin, after which the increase in concentration caused no additional increase in the intrinsic fluorescence due to tryptophan residues (Figure 5.6). The \textit{in vitro} activity data in primary adenoma and cancer samples showed 0.1 \(\mu\)M curcumin to be the most effective concentration overall for targeting sphere formation and this concentration was also more effective at reducing Nanog expression in Caco2 cells (Chapter 4). Therefore, current evidence supports the idea that 0.1 \(\mu\)M curcumin could actually affect the stability of Nanog by targeting Nanog phosphorylation. A cyclohexamide assay was performed to try and demonstrate directly that curcumin enhances the degradation of Nanog, however, the half-life for Nanog could not be determined in these cells because of the unexpected stability over the time frame concerned (8 h). Published literature indicates that Nanog might have a different pseudoform in cancer; in normal embryonic cells, Nanog exists as Nanog1 whereas in cancer, NanogP8 a product of a retrogene of Nanog1 is the major form [210]. Therefore, it is conceivable that differences between the Nanog forms in
cancer and normal embryonic cells may explain the variation in stability observed. However, such speculation requires extensive experimental verification.

Finally, the CD spectroscopy results are promising. The fact that the thermal stability of Nanog was reduced by the presence of 0.1 µM curcumin is consistent with structural changes in the protein conformation caused by the interaction between Nanog and curcumin.
6. Chapter 6: Concluding discussion

6.1. Cancer stem cells in the development of anticancer agents

The advent of the cancer stem cell hypothesis has provided the cancer research community with a novel potential route to develop anti-cancer drugs and chemopreventive agents, using an approach which differs from that used traditionally. “Classical” hypotheses associated with cancer development have engendered chemotherapeutic drugs that target specific neoplastic pathways or oncogenic molecules essential in driving carcinogenesis, thus ultimately leading to tumour regression. However, the drawbacks of this approach are considerable. Many chemotherapeutic agents possess severe side effects which compromise the quality of the patient’s life. Most importantly, many tumours treated with anticancer drugs, including recently developed molecular targeted agents (also now sometimes called “precision anticancer medicines”) acquire drug resistance. Thus, the cancer re-grows, often in a more aggressive form than the original malignancy. The development of anticancer agents, starting from pre-clinical evaluation to a phase III clinical trial, involves a huge investment. Therefore, researchers need to be confident at the preclinical stage of development that the experimental evidence to support anticancer efficacy is substantial - if not overwhelming - in order to justify further advancement to the clinical trial stage.

The work described in this thesis has used the cancer stem cell hypothesis as the basis for pre-clinical evaluation of curcumin in targeting stem-like cells in human colorectal cancer and adenoma. The first part of the project focussed on the selection of markers for identifying stem-like cells in colon cancer, using primary colon cancer and adenoma samples. This work led to the second phase of the study in which the efficacy of curcumin in targeting cancer stem-like cells both in vitro and in vivo was demonstrated using patient-derived colon samples. To my knowledge this is the first report of the activity of curcumin in primary human adenomas and the first time curcumin has been shown to target stem-like cells in primary human colorectal cancers. These insights support further assessment of the pre-clinical models employed to aid the optimal design of colon cancer prevention trials. Finally, a novel mechanism of action of curcumin in targeting cancer stem-like cells was delineated, which might also have the potential to be exploited in targeted anti-cancer drug development.
6.2. Searching for cancer stem cell markers

There are several markers of colorectal cancer stem-like cells reported to date. Among these are CD133, CD44 and CD24 [78] [89], however, their consistency and suitability have been queried in subsequent publications [89]. Although CD133 has been previously reported to be a reliable marker for cancer stem-like cells in colon cancer, others have shown that CD133 lacks specificity as its expression is not limited to a specific subset of cells and it is expressed in the majority of tumour cells irrespective of the stage of malignancy [211] [212]. Also, in my experience the CD133+ population failed to exhibit any phenotypic resemblance to cancer stem-like cells in vitro (refer to Chapter 3). It has been suggested that both CD44 and CD24 may serve as markers of cancer stem-like cells in colon cancer. However, the specificity of CD44 in identifying stemness is doubtful [211]. Recent publications suggest that loss of CD44 protein expression accompanies a poor clinical outcome [213] [214] [215], which implies that this marker might not be clinically relevant. Consequently, CD44 has not been used in the work described here to profile cancer stem-like cells in clinical samples. The evidence for CD24 as a marker of colonic cancer stem-like cells is scant [211], hence, CD24 has also not been used as a marker of colon cancer stem-like cells here.

Several publications suggest that high ALDH activity and/or expression is a marker of stem-like cells in various cancers. Huang et al. [89] demonstrated that ALDH is probably a better marker of stem-like cells than CD133 and CD44 in terms of specificity (Chapter 1). The data described in Chapter 3, which suggests ALDH activity is a more robust marker of stem-like cells in both cancer and adenoma samples than CD133 and CD44 expression, is in accordance with this judgement. There was also a correlation (R²=0.57) between ALDH activity and in vitro tumourigenic capacity in primary colon cancer cells, as described in Chapter 3.

The results of the in vivo experiments in which ALDH\textsuperscript{high/low} cells were injected into NOD/SCID mice (Chapter 3) are also consistent with the notion that ALDH activity may be a reliable marker of stemness in colorectal cancer. Serial passaging and implantation of tumours in the NOD/SCID mice did not lead to loss of this tumourigenic population of cells, as marked by ALDH\textsuperscript{high} activity, in vivo. It is important to note that once ALDH\textsuperscript{high} cells were injected into the NOD/SCID mice, the
resulting tumour did not represent a homogenous population of ALDH$^{\text{high}}$ cells, as there were also cells of other progeny. This finding raises the possibility that whilst the tumour-initiating capacity is rooted within the ALDH$^{\text{high}}$ subpopulation, ALDH$^{\text{high}}$ cells can also be the source of other cellular progenies within the tumour mass. This observation implies that the stem-like cells marked by ALDH activity could constitute the early progenitor cells in the stem cell hierarchy (see Chapter 3).

Based on all the deductions made from the profiling data, both ALDH activity and CD133 expression were used as markers for cancer stem-like cells in the subsequent efficacy studies \textit{in vitro} and \textit{in vivo}. The use of CD133 was considered to give an indication of whether this marker could be a possible target of intervention by curcumin, as CD133 positivity might represent a population of neoplastic cells that are committed towards differentiation.

\section*{6.3. Effects of curcumin}

A remarkable number of anti-cancer effects of curcumin have been reported (refer to Chapter 1). Curcumin targets numerous pathways in carcinogenesis to mediate its anti-cancer effects [40]. However, the effective concentration for most of these activities is in the range of 10–100 µM [31]. The work described in this thesis provides probably the first evidence for biological activity of curcumin \textit{in vitro} at submicromolar concentrations - concentrations which are clinically achievable in plasma and colorectal tissue [174]. Also of interest is the fact that a linear dose response curve was not observed here. In more than 50% of the \textit{in vitro} samples studied the response was U-shaped, indicating that the optimal effective concentration is in the submicromolar range, notably lower than the highest concentration (5 µM) used. A U-shaped dose response has also been demonstrated by other members of the Leicester group, particularly for resveratrol. Furthermore, published evidence suggests resveratrol has a biphasic dose response in cultured endothelial progenitor cells, with greater activity at the lower concentrations on a pathway relevant to carcinogenesis; at 60 nmol/L resveratrol decreased nitric oxide synthase expression, whereas at 1 µmol/L resveratrol increased the expression [23]. Therefore, the existence of biphasic dose responses
indicates that low concentrations of cancer preventive agents could potentially elicit more beneficial effects than high concentrations.

One may argue that the strength of the type of data obtained here with curcumin is derived from the fact that primary cells were used in the *in vitro* activity and *in vivo* efficacy studies. These preclinical models arguably represent the clinical situation more faithfully than established cultured cancer cell lines. However, there is considerable variability in the data described in Chapter 4. This inter-individual variation could be partly attributed to the fact that the primary samples used were randomly selected and they represent different clinical stages of colon cancer. In order to explore the reasons for differences in the dose-response relationships, more in-depth studies would be required using samples from a much larger cohort of patients with different clinical stages of disease and different driver mutations.

The *in vivo* efficacy data from NOD/SCID mice (Chapter 4) suggests a delay in the growth of tumours in mice maintained on a curcumin-containing diet. However, the sample size in both treatment and control arms is small. Also, this experiment was performed using just one primary tumour sample. The study is currently being repeated with samples from two other patients to confirm the findings described here. In the *Apc*<sup>Min</sup> mouse model, curcumin was also found to target cancer stem-like cells defined by DCAMKL-1 expression (Chapter 4). The lack of effect on DCAMKL-1<sup>+</sup> cells in C57/BL6 wild type animals that represent the background strain for *Apc*<sup>Min</sup> mice, further confirms that curcumin exclusively targets cancer or premalignant stem-like cells and not the normal stem cells.

### 6.4. Clinical opportunities for curcumin

The results presented here obtained from both experiments *in vitro* and *in vivo* present a strong case for curcumin as an agent which can target cancer stem-like cells in human colon cancer. This insight suggests it may be possible to design novel prevention or therapy regimens using curcumin as a single agent or in combination with other chemopreventive or established chemotherapeutic drugs. In the case of therapeutic trials, the combination of chemotherapeutic drugs with curcumin might increase the chance that chemotherapy-resistant cells, which are essentially the cancer stem-like
cells, will be targeted together with the differentiated progeny in the tumour mass, resulting in better clinical outcome. Currently, there are few clinical strategies that exploit a combination of drugs to target cancer stem-like cells. One such strategy involves Notch signalling, one of the regulators of stem cell self-renewal (see Chapter 1). This signalling system has been targeted by a novel combination treatment in the phase 1 clinical setting of pancreatic and breast cancer [216, 217]. The combination consisted of MK0752, an inhibitor of the Notch signalling component gamma-secretase with either gemcitabine in patients with stage III and IV pancreatic cancer, or tamoxifen or letrozole for early stage breast cancer. To date the results of these trials have not been published.

6.5. Nanog, a target of curcumin

To my knowledge, the results presented here are the first to describe a mechanism of action of curcumin involving Nanog. Nanog has been extensively studied in terms of its role in embryonic stem cell self-renewal, as outlined in Chapter 1. In recent years, overexpression of Nanog has been correlated with a poor clinical prognosis in oral, breast and colorectal cancer [218-220]. Most of the regulatory processes involving Nanog that have been described in the literature are at the transcriptional level. Only recently has the focus shifted to its post-transcriptional and translational regulation [221]. Although attempts detailed here failed to show any regulation of Nanog by curcumin at the transcriptional level, curcumin was found for the first time to be able to down-regulate Nanog protein levels and reduce the extent of phosphorylation. Subsequent studies indicated a direct interaction of curcumin with Nanog at the protein level (Chapter 5). These findings imply that curcumin might destabilize the Nanog protein, thereby decreasing its expression in the cancer stem-like population. A comparison between resveratrol and curcumin seems instructive in this context. Resveratrol failed to exhibit any direct interaction with Nanog, and showed no ability to target the cancer stem-like population when assessed using primary in vitro spheroid assays. In contrast, curcumin non-covalently bound to Nanog and targeted the cancer stem-like cell population, which suggests there may be a functional link. The biophysical data accrued subsequently are also promising in showing a potentially functionally-important interaction between curcumin and Nanog. Nevertheless, further
more comprehensive investigations are warranted to confirm the preliminary findings of this link.

Currently, efforts in our laboratory are attempting to unravel the sites within the Nanog protein to which curcumin can bind. Different fragments of the Nanog protein will be expressed, including peptides containing a DNA binding homeo domain, a tryptophan repeat fragment, a transactivation and an N-terminal domain. The aim of this work is to further verify curcumin binding and identify which domain(s) of the Nanog protein curcumin interacts with. Another important aspect is to explore the effect of curcumin on the downstream transcriptional targets of Nanog, which would further confirm that interaction between the two is functionally important. To this effect, a couple of direct targets of Nanog have been identified, including Bmi-1 [222] and Esrrb (estrogen related receptor b) [223], which are implicated in tumourigenesis and embryonic stem cell development, respectively. Once the functional relevance of the interaction between curcumin and Nanog has been established, elucidation of the molecular structure of the Nanog-curcumin complex will be attempted by NMR spectroscopy.

Overall, the project described in this thesis shows that curcumin can target human colorectal cancer stem-like cells both in vitro and in vivo. Crucially, the concentrations at which this efficacy was observed are clinically achievable. The mechanism of action of curcumin in mediating its inhibitory effects via cancer stem-like cells hints at a novel strategy towards the development of anti-cancer drugs targeted against Nanog.
Figure 7.1. Effect of curcumin on the expression of stem cell proteins in primary colorectal cancer sphere-derived cells. Single cells from three primary colorectal tumours were plated at a density of 100,000 cells/well in six well ultra-low attachment plates to allow sphere growth. Curcumin was added at respective concentrations twice weekly for 4 weeks. Whole spheres were then harvested, made into single cells and lysed. The expressions of proteins were assessed using a human pluripotent stem cell protein profiler kit (for details see Methods Section 2.12). Patients 1, 2 and 3 correspond to the same patient samples used to generate the data shown in Figure 4.10, Chapter 4.
Figure 7.2. Effect of resveratrol on sphere formation in 2 cancer and 1 adenoma patient sample. Single cells from primary colorectal tumours (A, B and D) and an adenoma (C) were plated at a density of 30,000 cells/well in six well ultra-low attachment plates. Resveratrol was added twice weekly for 4 weeks and following treatment, spheres were counted in two cancers (A&B) and one adenoma (C) patient. Sphere size was measured in one cancer sample (D). All data points on sphere numbers for both cancer and adenoma samples were duplicates, performed on a single experiment except for 0.01 and 1 µM concentration in Figure B where triplicate data (one single experiment) was available. Sphere size data are represented as the average sphere size of more than 25 (AVG ± SEM) spheres measured for each concentration on a single experiment.
Table 7.1. Age, Duke’s stage and gender of patient samples

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8. References


